U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
UEMURA 8

U.S. APPLICATION NO. (If known,see 37 CFR 1.5)
09/856050

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY CLAIMED
PCT/JP99/06474	19 November 1999	20 November 1998

TITLE OF INVENTION
PROTEIN EXPRESSION VECTOR AND UTILIZATION THEREOF

APPLICANT(S) FOR DO/EO/US
Hidetoshi UEMURA et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not transmitted by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

tems 11. to 16. below concern document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    [X] Courtesy copy of the first page of the International Publication (WO 00/31284).
    [X] Courtesy copy of the International Preliminary Examination Report (In Japanese). There were no annexes.
    [X] Formal drawings, 8 sheets, Figures 1-8.
    [X] Courtesy Copy of the International Search Report.
    [X] The application is (or will be) assigned to:**FUSO PHARMACEUTICAL INDUSTRIES, LTD.**, whose address is 7-10,)oshomachi 1-chome, Chuo-ku, Osaka-shi, Osaka Japan
    [X] Application Data Sheet

PLICATION NO. (If known,see 37 CFR 1.5)	International Application No.	Attorney's Docket No.
09/856050	PCT/JP99/06474	UEMURA 8

17. [xx] The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....**$1000.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....**$860.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....**$710.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....**$690.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....**$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

Claims as Originally Presented	Number Filed	Number Extra	Rate	
Total Claims	25 - 20	05	X $18.00	$ 90.00
Independent Claims	1 - 3		X $80.00	$
Multiple Dependent Claims (if applicable)			+$270.00	$ 270.00
TOTAL OF ABOVE CALCULATIONS =				$1,220.00

Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate	
Total Claims	- 20		X $18.00	$
Independent Claims	- 3		X $78.00	$
TOTAL OF ABOVE CALCULATIONS =				$1,220.00
Reduction of ½ for filing by small entity, if applicable. Applicant claims small entity status. See 37 CFR 1.27.				$
SUBTOTAL =				$1,220.00
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$
TOTAL NATIONAL FEE =				$1,220.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$1,220.00
			Amount to be: refunded	$
			charged	$

[ ] A check in the amount of $_____ to cover the above fees is enclosed.

[X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of $ 1,220.00, is attached.

[ ] Please charge my Deposit Account No. **02-4035** in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

[XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4035**. A duplicate copy of this sheet is enclosed.

OTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or)) must be filed and granted to restore the application to pending status.

ND ALL CORRESPONDENCE TO:

ROWDY AND NEIMARK, P.L.L.C.
4 NINTH STREET, N.W., SUITE 300
ASHINGTON, D.C. 20001
L: (202) 628-5197
X: (202) 737-3528
e of this submission: **May 17, 2001**

SIGNATURE

Roger L. Browdy
NAME

25,618
REGISTRATION NUMBER

#4

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: UEMURA8

In re Application of:	)	Art Unit:
H. UEMURA, et al.	)	Examiner:
Serial No.: 09/856,050	)	Confirmation No.
Filed: May 17, 2001	)	Washington D.C.
For: PROTEIN EXPRESSION VECTOR AND UTILIZATION THEREOF	)	August 17, 2001

RESPONSE TO NOTIFICATION TO COMPLY WITH REQUIREMENTS FOR PATENT APPLICATIONS CONTAINING NUCLEOTIDE SEQUENCE AND/OR AMINO ACID SEQUENCE DISCLOSURES

Honorable Commissioner for Patents
Washington, D.C. 20231

In response to the Notice to Comply, dated June 20, 2001, and prior to the examination of the above-described application, please amend the present application as follows:

IN THE SPECIFICATION

Please replace the paragraph beginning at page 9, line 6, with the following rewritten paragraph:

--(5) The protein expression vector according to the above (4), wherein the spacer nucleotide sequence is anucleotide sequence encoding at least the amino acid sequence of Leu-Val-His-Gly-Lys-Leu (amino acid 24-29 of SEQ ID NO:19);--

Please replace the second paragraph from the bottom of page 7, with the following rewritten paragraph:

--(8) The protein expression vector according to the above (7), wherein the cleavable nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Asp-Asp-Asp-Asp-Lys (amino acid 19-23 of SEQ ID NO:19);--

Please replace the paragraph beginning at page 20, line 11, with the following rewritten paragraph:

--For example, a nucleotide sequence encoding an amino acid sequence which is susceptible to enzyme-specific cleavage corresponds to this sequence. Examples thereof include as follows: a nucleotide sequence encoding the amino acid sequence of Asp-Asp-Asp-Asp-Lys (amino acid 19-23 of SEQ ID NO:19) (said amino acid sequence is recognized by enterokinase, and the recombinant fusion protein is cleaved at the C-terminus thereof); a nucleotide sequence encoding the amino acid sequence of Leu-Val-Pro-Arg-Gly-Ser (SEQ ID NO:20) (said amino acid sequence is recognized by thrombin, and the recombinant fusion protein is cleaved between Arg-Gly thereof); a nucleotide sequence encoding the amino acid sequence Ile-Glu-Gly-Arg (SEQ ID NO:21) (said amino acid sequence is recognized by factor Xa, and the recombinant fusion protein is cleaved at the C-terminus thereof); a nucleotide sequence encoding the amino acid sequence Glu-Asn-

Leu-Tyr-Phe-Gln (SEQ ID NO:22) (said amino acid sequence is recognized by TEV (Tobacco Etch virus) protease, and the recombinant fusion protein is cleaved at the C-terminus thereof), and the like.--

Please replace the paragraph beginning at page 23, line 6, with the following rewritten paragraph:

--A space sequence may be, for example, a cleavable sequence from which the secretory signal, the Tag sequence and epitope can be cleaved by enzyme, or the like. In particular, in the case where there is a histidine Tag upstream of the target protein, inserting successively a prepro-region in the secretory signal and inserting the amino acid sequence Leu-Val-His-Gly-Lys-Leu (amino acid 24-29 of SEQ ID NO:19) as a spacer sequence to the C-terminus of the prepro-region are convenient for the cleavage by an enzyme, or the like, because the distance between the trypsin signal and the histidine Tag becomes larger.--

Please replace the paragraph beginning at page 25, line 4, with the following rewritten paragraph:

--The following Examples further illustrate the present invention in detail but are not to be construed to limit the scope of the present invention. In the following Examples, IgGk leader may be understood as a synonym of the

secretory signal of IgG. When DDDDK (Asp-Asp-Asp-Asp-Lys) (amino acid 19-23 of SEQ ID NO:19) is present proximate to a trypsin signal, the DDDDK (amino acid 19-23 of SEQ ID NO:19) and the trypsin signal inclusive is called as trypsin signal in some cases (the sequence of 1st to 23rd amino acids in SEQ ID NO: 19), whereas only the trypsin signal without containing said DDDDK (amino acid 19-23 of SEQ ID NO:19) is as called trypsin signal (the sequence of 1st to 18th in SEQ ID NO:19) in other cases. Those skilled in the art can readily understand that a particular sequence corresponds to either of which from the context of the description. The trypsin signal shown in Figs. 1, 3 and 5 refers to the 1st to 18th amino acids in SEQ ID NO: 19. In this connection, IgGk signal and the trypsin signal may be used in an interchangeable manner and, in this ~~resepct~~ respect, both are considered to be equivalent, and the trypsin signal referred to herein may or may not include DDDDK.--

Please replace the first paragraph beginning at page 31, with the following rewritten paragraph:

--The portion of pSecTrypHis/Neurosin spanning from the trypsin signal to the enterokinase recognition site was amplified by using SEQ ID NOS: 10 and 11 such that the peptide Leu-Val-His-Gly (amino acid 1-4 of SEQ ID NO;15) was located at the C-terminus. The product was inserted between Nhe I and

Hind III sites of pSecTag2A to obtain the plasmid pTrypSig. About 200 bp which contained His tag region in pTrypHis was amplified by using SEQ ID NOS: 11 and 7. A fragment of about 40 bp containing His tag and enterokinase recognition site, which was produced by digesting with Hind III and BamH I, was inserted into pTrypSig to obtain pTrypSigTag (Fig. 5A).--

Please replace the paragraph beginning at the bottom of page 35, with the following rewritten paragraph:

--The protein expression vector of the present invention is advantageous and characterized by in that the protein expression vector has the above-described specific construction of the components thereby facilitating the purification and recovery of a target protein in a mature form or an active form. A preferred example of the construction of said protein expression vector includes a secretory signal nucleotide sequence, a Tag nucleotide sequence positioned in the 3' downstream thereof, a cleavable nucleotide sequence comprising a nucleotide sequence encoding the amino acid sequence of Asp-Asp-Asp-Asp-Lys <u>(amino acid 36-40 of SEQ ID NO:19)</u> capable of being recognized by enterokinase, a nucleotide sequence encoding the target protein positioned successively in the downstream, and a nucleotide sequence containing a stop codon positioned in the furthest downstream, where it is possible by using this vector to produce a

recombinant protein without additional amino acids attached to the N-terminus or the C-terminus of the target protein, namely the target protein of a mature form or an active form.--

IN THE CLAIMS

Please replace claims 5 and 8 with new claims 5 and 8 as follows below. A marked up version of the amended claims is attached hereto.

5. The protein expression vector according to claim 4, wherein the spacer nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Leu-Val-His-Gly-Lys-Leu (amino acid 24-29 of SEQ ID NO:19).

8. The protein expression vector according to claim 7, wherein the cleavable nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Asp-Asp-Asp-Asp-Lys (amino acid 36-40 of SEQ ID NO:19).

IN THE SEQUENCE LISTING

Please enter the attached Sequence Listing, numbered as pages 1-9.

REMARKS

Applicants have added into the present specification a new paper copy Sequence Listing section according to 37 C.F.R. §1.821(c) as new pages 1-9, and have renumbered the

subsequent pages accordingly. Furthermore, attached hereto is a 3 1/2" disk containing the "Sequence Listing" in computer readable form in accordance with 37 C.F.R. §1.821(e).

Applicants have amended the specification to insert SEQ ID Nos, as supported in the present specification.

The following statement is provided to meet the requirements of 37 C.F.R. §1.825(a) and 1.825(b).

I hereby state, in accordance with 37 C.F.R. §1.825(a), that the amendments included in the substitute sheets of the sequence listing are believed to be supported in the application as filed and that the substitute sheets of the sequence listing are not believed to include new matter.

I hereby further state, in accordance with 37 C.F.R. §1.825(b), that the attached copy of the computer readable form is the same as the attached substitute paper copy of the sequence listing.

Under U.S. rules, each sequence must be classified in <213> as an "Artificial Sequence", a sequence of "Unknown" origin, or a sequence originating in a particular organism, identified by its scientific name.

Neither the rules nor the MPEP clarify the nature of the relationship which must exist between a listed sequence and an organism for that organism to be identified as the origin of the sequence under <213>.

Hence, counsel may choose to identify a listed

sequence as associated with a particular organism even though that sequence does not occur in nature by itself in that organism (it may be, e.g., an epitopic fragment of a naturally occurring protein, or a cDNA of a naturally occurring mRNA, or even a substitution mutant of a naturally occurring sequence). Hence, the identification of an organism in <213> should not be construed as an admission that the sequence *per se* occurs in nature in said organism.

Similarly, designation of a sequence as "artificial" should not be construed as a representation that the sequence has no association with any organism. For example, a primer or probe may be designated as "artificial" even though it is necessarily complementary to some target sequence, which may occur in nature. Or an "artificial" sequence may be a substitution mutant of a natural sequence, or a chimera of two or more natural sequences, or a cDNA (i.e., intron-free sequence) corresponding to an intron-containing gene, or otherwise a fragment of a natural sequence.

The Examiner should be able to judge the relationship of the enumerated sequences to natural sequences by giving full consideration to the specification, the art cited therein, any further art cited in an IDS, and the results of his or her sequence search against a database containing known natural sequences.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

Applicants submit that the present application contains patentable subject matter and therefore urge the examiner to pass the case to issuance.

If the examiner has any questions or comments concerning the above described application, the examiner is urged to contact the undersigned at the phone number below.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By _____
Allen C. Yun
Registration No. 37,971

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624 Ninth Street, N.W.
Washington, D.C. 20001
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528
F:\,A\Aoyb\Uemura 8\PTO\notice to comply with seq.wpd

VERSION WITH MARKINGS TO SHOW THE CHANGES MADE

The paragraph beginning at page 9, line 6, has been rewritten as follows:

(5) The protein expression vector according to the above (4), wherein the spacer nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Leu-Val-His-Gly-Lys-Leu (amino acid 24-29 of SEQ ID NO:19);

The second paragraph from the bottom of page 7, has been rewritten as follows:

(8) The protein expression vector according to the above (7), wherein the cleavable nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Asp-Asp-Asp-Asp-Lys (amino acid 19-23 of SEQ ID NO:19);

The paragraph beginning at page 20, line 11, has been rewritten as follows:

For example, a nucleotide sequence encoding an amino acid sequence which is susceptible to enzyme-specific cleavage corresponds to this sequence. Examples thereof include as follows: a nucleotide sequence encoding the amino acid sequence of Asp-Asp-Asp-Asp-Lys (amino acid 19-23 of SEQ ID NO:19) (said amino acid sequence is recognized by enterokinase, and the recombinant fusion protein is cleaved at the C-terminus thereof); a nucleotide sequence encoding the amino acid

sequence of Leu-Val-Pro-Arg-Gly-Ser (SEQ ID NO:20) (said amino acid sequence is recognized by thrombin, and the recombinant fusion protein is cleaved between Arg-Gly thereof); a nucleotide sequence encoding the amino acid sequence Ile-Glu-Gly-Arg (SEQ ID NO:21) (said amino acid sequence is recognized by factor Xa, and the recombinant fusion protein is cleaved at the C-terminus thereof); a nucleotide sequence encoding the amino acid sequence Glu-Asn-Leu-Tyr-Phe-Gln (SEQ ID NO:22) (said amino acid sequence is recognized by TEV (Tobacco Etch virus) protease, and the recombinant fusion protein is cleaved at the C-terminus thereof), and the like.

The paragraph beginning at page 23, line 6, has been rewritten as follows:

--A space sequence may be, for example, a cleavable sequence from which the secretory signal, the Tag sequence and epitope can be cleaved by enzyme, or the like. In particular, in the case where there is a histidine Tag upstream of the target protein, inserting successively a prepro-region in the secretory signal and inserting the amino acid sequence Leu-Val-His-Gly-Lys-Leu (amino acid 24-29 of SEQ ID NO:19) as a spacer sequence to the C-terminus of the prepro-region are convenient for the cleavage by an enzyme, or the like, because the distance between the trypsin signal and the histidine Tag becomes larger.--

The paragraph beginning at page 25, line 4, has been amended as follows:

The following Examples further illustrate the present invention in detail but are not to be construed to limit the scope of the present invention. In the following Examples, IgGk leader may be understood as a synonym of the secretory signal of IgG. When DDDDK (Asp-Asp-Asp-Asp-Lys) (amino acid 19-23 of SEQ ID NO:19) is present proximate to a trypsin signal, the DDDDK (amino acid 19-23 of SEQ ID NO:19) and the trypsin signal inclusive is called as trypsin signal in some cases (the sequence of 1st to 23rd amino acids in SEQ ID NO: 19), whereas only the trypsin signal without containing said DDDDK (amino acid 19-23 of SEQ ID NO:19) is as called trypsin signal (the sequence of 1st to 18th in SEQ ID NO:19) in other cases. Those skilled in the art can readily understand that a particular sequence corresponds to either of which from the context of the description. The trypsin signal shown in Figs. 1, 3 and 5 refers to the 1st to 18th amino acids in SEQ ID NO: 19. In this connection, IgGk signal and the trypsin signal may be used in an interchangeable manner and, in this ~~resepct~~ respect, both are considered to be equivalent, and the trypsin signal referred to herein may or may not include DDDDK.

The first paragraph beginning at page 31, has been amended as follows:

The portion of pSecTrypHis/Neurosin spanning from the trypsin signal to the enterokinase recognition site was amplified by using SEQ ID NOS: 10 and 11 such that the peptide Leu-Val-His-Gly (amino acid 1-4 of SEQ ID NO;15) was located at the C-terminus. The product was inserted between Nhe I and Hind III sites of pSecTag2A to obtain the plasmid pTrypSig. About 200 bp which contained His tag region in pTrypHis was amplified by using SEQ ID NOS: 11 and 7. A fragment of about 40 bp containing His tag and enterokinase recognition site, which was produced by digesting with Hind III and BamH I, was inserted into pTrypSig to obtain pTrypSigTag (Fig. 5A).

The paragraph beginning at the bottom of page 35, has been amended as follows:

The protein expression vector of the present invention is advantageous and characterized by in that the protein expression vector has the above-described specific construction of the components thereby facilitating the purification and recovery of a target protein in a mature form or an active form. A preferred example of the construction of said protein expression vector includes a secretory signal nucleotide sequence, a Tag nucleotide sequence positioned in the 3′ downstream thereof, a cleavable nucleotide sequence comprising a nucleotide sequence encoding the amino acid sequence of Asp-Asp-Asp-Asp-Lys (amino acid 36-40 of SEQ ID

NO:19) capable of being recognized by enterokinase, a nucleotide sequence encoding the target protein positioned successively in the downstream, and a nucleotide sequence containing a stop codon positioned in the furthest downstream, where it is possible by using this vector to produce a recombinant protein without additional amino acids attached to the N-terminus or the C-terminus of the target protein, namely the target protein of a mature form or an active form.

IN THE CLAIMS

Claims 5 and 8 have been amended as follows:

5. The protein expression vector according to claim 4, wherein the spacer nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Leu-Val-His-Gly-Lys-Leu (amino acid 24-29 of SEQ ID NO:19).

8. The protein expression vector according to claim 7, wherein the cleavable nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Asp-Asp-Asp-Asp-Lys (amino acid 36-40 of SEQ ID NO:19).

SEQUENCE LISTING

<110> UEMURA, Hidetoshi
OKUI, Akira
KOMINAMI, Katsuya
YAMAGUCHI, Nozomi
MITSUI, Shinichi

<120> PROTEIN EXPRESSION VECTOR AND USE THEREOF

<130> UEMURA=8

<140> 09/856,050
<141> 2001-05-17

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gtc ttc ctg ggg aag cat aac ctt cgg caa agg gag agt tcc cag gag 192
Val Phe Leu Gly Lys His Asn Leu Arg Gln Arg Glu Ser Ser Gln Glu
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85 90 95

ctc tct gaa ctc atc cag ccc ctt ccc ctg gag agg gac tgc tca gcc 336
Leu Ser Glu Leu Ile Gln Pro Leu Pro Leu Glu Arg Asp Cys Ser Ala
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Asn Thr Thr Ser Cys His Ile Leu Gly Trp Gly Lys Thr Ala Asp Gly
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180 185 190

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Val Phe Leu Gly Lys His Asn Leu Arg Gln Arg Glu Ser Ser Gln Glu
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Gln Ser Ser Val Val Arg Ala Val Ile His Pro Asp Tyr Asp Ala Ala
65 70 75 80

Ser His Asp Gln Asp Ile Met Leu Leu Arg Leu Ala Arg Pro Ala Lys
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Leu Ser Glu Leu Ile Gln Pro Leu Pro Leu Glu Arg Asp Cys Ser Ala
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Asn Thr Thr Ser Cys His Ile Leu Gly Trp Gly Lys Thr Ala Asp Gly
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VW

PCT09

RAW SEQUENCE LISTING DATE: 10/11/2001
PATENT APPLICATION: **US/09/856,050** TIME: 09:50:56

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<110> APPLICANT: UEMURA, Hidetoshi
OKUI, Akira
KOMINAMI, Katsuya
YAMAGUCHI, Nozomi
MITSUI, Shinichi
<120> TITLE OF INVENTION: PROTEIN EXPRESSION VECTOR AND USE THEREOF
<130> FILE REFERENCE: UEMURA=8
<140> CURRENT APPLICATION NUMBER: 09/856,050
<141> CURRENT FILING DATE: 2001-05-17
<150> PRIOR APPLICATION NUMBER: JP 10/331515
<151> PRIOR FILING DATE: 1998-11-20
<150> PRIOR APPLICATION NUMBER: PCT/JP99/06474
<151> PRIOR FILING DATE: 1999-11-19
<160> NUMBER OF SEQ ID NOS: 22
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RAW SEQUENCE LISTING DATE: 10/11/2001
PATENT APPLICATION: US/09/856,050 TIME: 09:50:56

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RAW SEQUENCE LISTING DATE: 10/11/2001
PATENT APPLICATION: US/09/856,050 TIME: 09:50:56

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RAW SEQUENCE LISTING DATE: 10/11/2001
PATENT APPLICATION: **US/09/856,050** TIME: 09:50:56

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276             20                  25                  30
278 cca ctg tgg gtc ctc aca gct gcc cac tgc aaa aaa ccg aat ctt cag      144
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280         35                  40                  45
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284     50                  55                  60
286 cag agt tct gtt gtc cgg gct gtg atc cac cct gac tat gat gcc gcc      240
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288 65                  70                  75                  80
290 agc cat gac cag gac atc atg ctg ttg cgc ctg gca cgc cca gcc aaa      288
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RAW SEQUENCE LISTING DATE: 10/11/2001
PATENT APPLICATION: **US/09/856,050** TIME: 09:50:56

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300         115                 120                 125
302 gat ttc cct gac acc atc cag tgt gca tac atc cac ctg gtg tcc cgt      432
303 Asp Phe Pro Asp Thr Ile Gln Cys Ala Tyr Ile His Leu Val Ser Arg
304     130                 135                 140
306 gag gag tgt gag cat gcc tac cct ggc cag atc acc cag aac atg ttg      480
307 Glu Glu Cys Glu His Ala Tyr Pro Gly Gln Ile Thr Gln Asn Met Leu
308 145                 150                 155                 160
310 tgt gct ggg gat gag aag tac ggg aag gat tcc tgc cag ggt gat tct      528
311 Cys Ala Gly Asp Glu Lys Tyr Gly Lys Asp Ser Cys Gln Gly Asp Ser
312                 165                 170                 175
314 ggg ggt ccg ctg gta tgt gga gac cac ctc cga ggc ctt gtg tca tgg      576
315 Gly Gly Pro Leu Val Cys Gly Asp His Leu Arg Gly Leu Val Ser Trp
316             180                 185                 190
318 ggt aac atc ccc tgt gga tca aag gag aag cca gga gtc tac acc aac      624
319 Gly Asn Ile Pro Cys Gly Ser Lys Glu Lys Pro Gly Val Tyr Thr Asn
320         195                 200                 205
322 gtc tgc aga tac acg aac tgg atc caa aaa acc att cag gcc aag tga      672
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346         35                  40                  45
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350     50                  55                  60
353 Gln Ser Ser Val Val Arg Ala Val Ile His Pro Asp Tyr Asp Ala Ala
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357 Ser His Asp Gln Asp Ile Met Leu Leu Arg Leu Ala Arg Pro Ala Lys
358                 85                  90                  95
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VERIFICATION SUMMARY DATE: 10/11/2001
PATENT APPLICATION: **US/09/856,050** TIME: 09:50:57

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#3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: UEMURA8

In re Application of:	)	Art Unit:
H. UEMURA, et al.	)	Examiner:
Serial No.: 09/856,050	)	Confirmation No.
Filed: May 17, 2001	)	Washington D.C.
For: PROTEIN EXPRESSION VECTOR AND UTILIZATION THEREOF	)	August 3, 2001

SECOND PRELIMINARY AMENDMENT

Honorable Commissioner for Patents
Washington, D.C. 20231

Prior to examination of the present application, please enter the following Preliminary Amendment:

IN THE SPECIFICATION

Page 7, please amend the first paragraph as follows:

The present invention provides an expression vector which, upon insertion into various host cells (particularly animal cells such as mammalian cells and insect cells), can secrete a recombinant protein produced extracellularly, allows the simple purification of the produced recombinant protein, and still further provides the recombinant protein almost identical in quality to the natural protein. The expression vector provided herein may also be used in situations where it is preferred to use microorganisms and the like as the host, for example, where the presence of sugar chains on the protein

is not necessary, or protein production is carried out as a basic study.

Page 15, please amend the first paragraph as follows:

After translation, an active protein may be obtained. Even when the resultant protein is not an active protein, it may be converted to an active protein by a variety of techniques. In many cases, a protein is first synthesized at the ribosomes in the cytoplasm as an inactive precursor (pro-form) which comprises an active protein bearing at the N-terminus thereof a peptide of about 15 to 60 amino acids responsible for secretion (secretory signal). The peptide region, which functions as a secretory signal, is concerned with the mechanism of passing through the cell membrane, and is removed by cleavage with a specific protease during the passage through the membrane (not always) to yield a mature protein. The peptide moiety which functions as a secretory signal has a broad hydrophobic region comprising hydrophobic amino acids in the middle of the sequence, and basic amino acid residues at a site close to the N-terminus. A secretory signal may be understood as a synonym of a signal peptide.

Page 15, please amend the second paragraph as follows:

In addition, in some proteins, a peptide moiety which functions as a secretory signal is further attached to the N-terminus of an inactive precursor (pro-form), and such a protein is called as a prepro-protein (the prepro-form). For example, trypsin is present as a prepro-form immediately after translation into amino acids, as a pro-form after being secreted from cells, and is converted into active trypsin in the duodenum upon limited degradation by enteropeptidase or by self degradation. A pro-form from which an active protein region has been deleted is called a pro-region, a prepro-form from which a pro-form region has been deleted is called a pre-region, and a prepro-form from which an active protein region has been deleted is called a prepro-region.

Page 16, please amend the first paragraph as follows:

The "secretory signal nucleotide sequence", which is one of the essential components of the protein expression vector of the present invention, refers to the nucleotide sequence encoding a secretory signal. Also, the "secretory signal" refers to the pro-region when a protein is expressed as a pro-form, and at least the pre-region or the prepro-region when a protein is expressed as a prepro-form. However, the secretory signal is not limited in so far as it is capable of secreting the intracellularly expressed protein,

extracellularly. The secretory signal nucleotide sequence constructed within the protein expression vector of the present invention preferably encodes a secretory signal with a cleavage site at the C-terminus of the signal. When the sequence encodes a secretory signal that does not contain a cleavage site at the C-terminus, it is preferred to newly insert a nucleotide sequence encoding a cleavable site at the 3′ end of said secretory signal nucleotide sequence. This is, for example, a trypsin signal represented by 1st to 23rd amino acids in SEQ ID NO: 19. At the C-terminus (19th to 23rd amino acids) of said sequence, there is Asp-Asp-Asp-Asp-Lys which is recognizable by enterokinase.

Page 17, please amend the first paragraph as follows:

Since the secretory signals of eukaryotic cells are similar to those of prokaryotic cells, *Escherichia coli* and the like may be used as the host. Since the secretory signal has different extracellular secretory activities depending on the host, it is necessary to select a secretory signal appropriate to the host. Specific examples of secretory signals include IgG (κ) (or IgGk) signal (or leader) and trypsin signal, which exhibit particularly high secretory activities when insect cells or mammalian cells are used as the host cells. Other examples of secretory signals include

BiP of flies (*Drosophila*), melitin of honeybees, α-factor of Pichia pastoris, PHO, and the like. When a trypsin signal is referred to herein, it may be constructed by either the 1st to 18th amino acids or the 1st to 23rd amino acids in SEQ ID NO: 19. Further, the secretory signal also includes, other than those exemplified above, their homologs and variants which are capable of secreting proteins extracellularly.

Page 17, please amend the second paragraph as follows:

The "Tag nucleotide sequence", which is another essential component of the protein expression vector of the present invention, refers to a nucleotide sequence that encodes a Tag sequence. The "Tag sequence" refers to an amino acid sequence that is not derived from the nucleic acid encoding a target protein and is inserted in order to facilitate, when expressed, isolation, purification and recognition of the target protein. Therefore, such a Tag sequence may be, for example, an antigen or an epitope recognizable by an antibody. By retaining the recombinant fusion protein containing a Tag sequence in a substance capable of recognizing said Tag sequence, isolation and purification can be carried out easily.

Page 24, please amend the first paragraph as

follows:

Introduction of the above expression vectors into the host cells per se may be conducted by employing a conventional method which includes, for example, transfection by the lipopolyamine method, the DEAE-dextran method, Hanahan's method, the lipofectin method, the calcium phosphate method, microinjection, electroporation, and the like.

Page 26, please amend the second paragraph as follows:

Plasmid pSecTag2A (1 μg, 0.1 μl) was treated with the restriction enzymes Nhe I and BamH I to completely remove the region encoding IgGk leader sequence. To this solution were added 100 pmoles each of the sense DNA and the antisense DNA described above, and the mixture was treated at 70°C for 10 minutes, after which it was left standing at room temperature for 30 minutes to allow annealing. To 1 μl each of the His secretory signal sequence, which had been treated with Nhe I and BamH I, and pSecTag2A was added 2.0 μl of solution I of DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.), and the mixture was allowed to react at 16°C for 30 minutes. To the reaction mixture was added 0.1 ml of competent *Escherichia coli* cells XL1-Blue (Stratagene Company), and the mixture was allowed to react on ice for 30 minutes, followed by heat shock

at 42°C for 60 seconds. After the reaction mixture was left on ice for 2 minutes, 0.9 ml of the SOC medium (Toyobo Co., Ltd.) was added and the cells were shake-cultured at 37°C for one hour. The culture was centrifuged at 5,000 rpm for one minute and the supernatant was discarded. The sedimented competent cells were suspended in the solution remaining in the centrifugation tube, and applied to two ampicillin LB plates containing 100 μg/ml ampicillin at a ratio of 1 : 10. The cells were cultivated overnight at 37°C and, from plasmids obtained from the resulting colonies, those with inserted DNA of the His secretory signal were selected by PCR and designated as pTrypHis.

IN THE CLAIMS

Please amend claim 3 as follows:

3. (Amended) The protein expression vector according to claim 1, wherein the cloning site or the nucleotide sequence encoding the target protein is present successively at the 3′ end of the cleavable nucleotide sequence.

Please amend claim 4 as follows:

4. (Amended) The protein expression vector according to claim 1, wherein a nucleotide sequence encoding at least one amino acid is contained as a spacer nucleotide

sequence in the 3′ downstream side of the secretory signal nucleotide sequence, but in the 5′ upstream side of the cleavable nucleotide sequence.

Please amend claim 6 as follows:

6. (Amended) The protein expression vector according to claim 4, wherein the spacer nucleotide sequence is composed of at least a cleavable nucleotide sequence.

Please amend claim 7 as follows:

7. (Amended) The protein expression vector according to claim 1, wherein the cleavable nucleotide sequence, when translated into an amino acid sequence, is cleaved by an enzyme at immediate upstream and/or immediate downstream and/or in the middle of said amino acid sequence.

Please amend claim 9 as follows:

9. (Amended) The protein expression vector according to claim 7, wherein the enzyme is enterokinase.

Please amend claim 10 as follows:

10. (Amended) The protein expression vector according to claim 1, wherein the secretory signal nucleotide sequence is an IgG (κ) signal or a trypsin signal.

Please amend claim 11 as follows:

11. (Amended) The protein expression vector according to claim 1, wherein the Tag nucleotide sequence is polyhistidine.

Please amend claim 12 as follows:

12. (Amended) The protein expression vector according to claim 1, further comprising a nucleotide sequence encoding an antibody recognition epitope.

Please amend claim 13 as follows:

13. (Amended) The protein expression vector according to claim 1, wherein the nucleotide sequence encoding the target protein is that encoding neurosin.

Please amend claim 14 as follows:

14. (Amended) Host cells transformed with the protein expression vector according to claim 1.

Please amend claim 18 as follows:

18. (Amended) A process for producing a target protein which comprises using the protein expression vector according to claim 1.

Please amend claim 20 as follows:

20. (Amended) A process for producing a recombinant fusion protein comprising an amino acid sequence of a target

protein which comprises using the protein expression vector or the host cells according to claim 1.

Please amend claim 22 as follows:

22. (Amended) A process for producing a target protein which comprises retaining the recombinant fusion protein according to claim 21 with a substance capable of recognizing at least one of Tag and an epitope in said recombinant fusion protein, liberating the recombinant fusion protein from the substance to purify it, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant fusion protein, followed by collecting the released target protein.

Please amend claim 23 as follows:

23. (Amended) A process for producing a target protein, which comprises retaining the recombinant fusion protein according to claim 21 with a substance capable of recognizing at least one of Tag and an epitope in said recombinant fusion protein, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant fusion protein, followed by collecting the released target protein.

Please amend claim 24 as follows:

24. (Amended) A target protein is obtained by the process according to claim 22.

Please enter the following new claims:

25. (New) A process for producing a target protein comprising cultivating host cells according to claim 14.

26. (New) A target protein obtained by the process according to claim 25.

27. (New) A process for producing a recombinant fusion protein comprising an amino acid sequence of a target protein which comprises cultivating the host cells according to claim 1.

28. (New) A recombinant fusion protein comprising the amino acid sequence of the target protein obtained by the process according to claim 27.

29. (New) A target protein which is obtained by the process according to claim 23.

REMARKS

The present Preliminary Amendment is submitted in order to correct some self-evident typographical errors and to eliminate multiple dependencies.

It is respectfully submitted that the claims are in condition for examination, and prompt and favorable action are earnestly solicited.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
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By
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AMK:nmp
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"Version with markings to show changes"

IN THE SPECIFICATION

Page 7, please amend the first paragraph as follows:

The present invention provides an expression vector which, upon ~~using in~~ **insertion into** various host cells (particularly animal cells such as mammalian cells and insect cells), can secrete a recombinant protein produced extracellularly, allows the simple purification of the produced recombinant protein, and still further provides the recombinant protein almost identical in quality to the natural protein. The expression vector provided herein may also be used in situations where **it is preferred** to use microorganisms and the like as the host ~~is preferred~~, for example, where the presence of sugar chains on the protein is not necessary, or protein production is carried out as a basic study.

Page 15, please amend the first paragraph as follows:

After translation, an active protein may be obtained. Even when the resultant protein is not an active protein, it may be converted to an active protein by ~~applying~~ a variety of ~~processing~~ **techniques**. In many cases, a protein is first synthesized at the ribosomes in the cytoplasm as an inactive precursor (pro-form) which comprises an active protein bearing at the N-terminus thereof a peptide of about

15 to 60 amino acids responsible for secretion (secretory signal). The peptide region, which functions as a secretory signal, is concerned with the mechanism of passing through the cell membrane, and is removed by cleavage with a specific protease during the passage through the membrane (not always) to yield a mature protein. The peptide moiety which functions as a secretory signal has a broad hydrophobic region comprising hydrophobic amino acids in the middle of the sequence, and basic amino acid residues at a site close to the N-terminus. A secretory signal may be understood as a synonym of a signal peptide.

Page 15, please amend the second paragraph as follows:

In addition, in some proteins, a peptide moiety which functions as a secretory signal is further attached to the N-terminus of an inactive precursor (pro-form), and such a protein is called as a prepro-protein (the prepro-form). For example, trypsin is present as a prepro-form immediately after translation into amino acids, as a pro-form after being secreted from cells, and is converted into active trypsin in the duodenum upon limited degradation by enteropeptidase or by self degradation. A pro-form from which an active protein region has been deleted is called ~~as~~ a pro-region, a prepro-form from which a pro-form region has been deleted is called

~~as~~ a pre-region, and a prepro-form from which an active protein region has been deleted is called ~~as~~ a prepro-region.

Page 16, please amend the first paragraph as follows:

The "secretory signal nucleotide sequence", which is one of the essential components of the protein expression vector of the present invention, refers to the nucleotide sequence encoding a secretory signal. Also, the "secretory signal" refers to the pro-region when a protein **is** expressed as a pro-form, and at least the pre-region or the prepro-region when a protein **is** expressed as a prepro-form. However, the secretory signal is not limited in so far as it is capable of secreting the intracellularly expressed protein, extracellularly. The secretory signal nucleotide sequence constructed within the protein expression vector of the present invention preferably encodes a secretory signal with a cleavage site at the C-terminus of the signal. When the sequence encodes a secretory signal that does not contain a cleavage site at the C-terminus, it is preferred to newly insert a nucleotide sequence encoding a cleavable site at the 3' end of said secretory signal nucleotide sequence. This is, for example, a trypsin signal represented by 1st to 23rd amino acids in SEQ ID NO: 19. At the C-terminus (19th to 23rd amino acids) of said sequence, there is Asp-Asp-Asp-Asp-Lys which is

recognizable by enterokinase.

Page 17, please amend the first paragraph as follows:

Since the secretory signals of eukaryotic cells are similar to those of prokaryotic cells, *Escherichia coli* and the like may be used as the host. Since the secretory signal has different extracellular secretory activities depending on the host, it is necessary to select a secretory signal appropriate to the host. Specific examples of secretory signals include IgG (κ) (or IgGk) signal (or leader) and trypsin signal, which exhibit particularly high secretory activities when insect cells or mammalian cells are used as the host cells. Other examples of secretory signals include BiP of flies (*Drosophila*), melitin of honeybees, α-factor of Pichia pastoris, PHO, and the like. When **a** trypsin signal is referred **to** herein, it may be constructed by either **the** 1st to 18th amino acids or **the** 1st to 23rd amino acids in SEQ ID NO: 19. Further, the secretory signal also includes, other than those exemplified above, their homologs and variants which are capable of secreting proteins extracellularly.

Page 17, please amend the second paragraph as follows:

The "Tag nucleotide sequence", which is another essential component of the protein expression vector of the present invention, refers to a nucleotide sequence that encodes a Tag sequence. The "Tag sequence" refers to an amino acid sequence that is ~~no~~ not derived from the nucleic acid encoding a target protein and is inserted in order to facilitate, when expressed, isolation, purification and recognition of the target protein. Therefore, such a Tag sequence may be, for example, an antigen or an epitope recognizable by an antibody. By retaining the recombinant fusion protein containing a Tag sequence in a substance capable of recognizing said Tag sequence, isolation and purification can be carried out easily.

Page 24, please amend the first paragraph as follows:

Introduction of the above expression vectors into the host cells per se may be conducted by employing ~~one of~~ a conventional ~~methods~~ method which ~~include~~ includes, for example, transfection by the lipopolyamine method, the DEAE-dextran method, Hanahan's method, the lipofectin method, ~~and~~ the calcium phosphate method, microinjection, electroporation, and the like.

Page 26, please amend the second paragraph as follows:

Plasmid pSecTag2A (1 μg, 0.1 μl) was treated with the restriction enzymes Nhe I and BamH I to completely remove the region encoding IgGk leader sequence. To this solution were added 100 pmoles each of the sense DNA and the antisense DNA described above, and the mixture was treated at 70°C for 10 minutes, after which it was left standing at room temperature for 30 minutes to allow annealing. To 1 μl each of the His secretory signal sequence, which had been treated with Nhe I and BamH I, and pSecTag2A was added 2.0 μl of solution I of DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.), and the mixture was allowed to react at 16°C for 30 minutes. To the reaction mixture was added 0.1 ml of competent *Escherichia coli* cells XL1-Blue (Stratagene Company), and the mixture was allowed to react on ice for 30 minutes, followed by heat shock at 42°C for 60 seconds. After the reaction mixture was left on ice for 2 minutes, 0.9 ml of the SOC medium (Toyobo Co., Ltd.) was added and the cells were shake-cultured at 37°C for one hour. The culture was centrifuged at 5,000 rpm for one minute and the supernatant was discarded. The sedimented competent cells ~~was~~ **were** suspended in the solution remaining in the centrifugation tube, and applied to two ampicillin LB plates containing 100 μg/ml ampicillin at a ratio of 1 : 10. The

cells were cultivated overnight at 37°C and, from plasmids obtained from the resulting colonies, those with inserted DNA of the His secretory signal were selected by PCR and designated as pTrypHis.

IN THE CLAIMS

3. (Amended) The protein expression vector according to claim 1 ~~or 2~~, wherein the cloning site or the nucleotide sequence encoding the target protein is present successively at the 3′ end of the cleavable nucleotide sequence.

4. (Amended) The protein expression vector according to ~~any one of claims 1 to 3~~ claim 1, wherein a nucleotide sequence encoding at least ~~on~~ one amino acid is contained as a spacer nucleotide sequence in the 3′ downstream side of the secretory signal nucleotide sequence, but in the 5′ upstream side of the cleavable nucleotide sequence.

6. (Amended) The protein expression vector according to claim 4 ~~or 5~~, wherein the spacer nucleotide sequence is composed of at least a cleavable nucleotide sequence.

7. (Amended) The protein expression vector

according to ~~any one of claims 1 to 6~~ claim 1, wherein the cleavable nucleotide sequence, when translated into an amino acid sequence, is cleaved by an enzyme at immediate upstream and/or immediate downstream and/or in the middle of said amino acid sequence.

9. (Amended) The protein expression vector according to claim 7 ~~or 8~~, wherein the enzyme is enterokinase.

10. (Amended) The protein expression vector according to ~~any one of claims 1 to 9~~ claim 1, wherein the secretory signal nucleotide sequence is an IgG (κ) signal or a trypsin signal.

11. (Amended) The protein expression vector according to ~~any one of claims 1 to 10~~ claim 1, wherein the Tag nucleotide sequence is polyhistidine.

12. (Amended) The protein expression vector according to ~~any one of claims 1 to 11~~ claim 1, further comprising a nucleotide sequence encoding an antibody recognition epitope.

13. (Amended) The protein expression vector according to ~~any one of claims 1 to 12~~ claim 1, wherein the nucleotide sequence encoding the target protein is that

encoding neurosin.

14. (Amended) Host cells transformed with the protein expression vector according to ~~any one of claims 1 to 13~~ claim 1.

18. (Amended) A process for producing a target protein which comprises using the protein expression vector ~~or the host cells~~ according to ~~any one of claims 1 to 18~~ claim 1.

20. (Amended) A process for producing a recombinant fusion protein comprising an amino acid sequence of a target protein which comprises using the protein expression vector or the host cells according to ~~any one of claims 1 to 18~~ claim 1.

22. (Amended) A process for producing a target protein which comprises retaining the recombinant fusion protein according to claim 21 with a substance capable of recognizing at least one of Tag and~~/or~~ an epitope in said recombinant fusion protein, liberating the recombinant fusion protein from the substance to purify it, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant fusion protein, followed by collecting the released target protein.

23. (Amended) A process for producing a target protein, which comprises retaining the recombinant fusion protein according to claim 21 with a substance capable of recognizing **at least one of** Tag and~~/or~~ an epitope in said recombinant fusion protein, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant fusion protein, followed by collecting the released target protein.

24. (Amended) A target protein is obtained by the process according to claim 22~~ or 23~~.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: UEMURA 8

In re Application of: Hidetoshi UEMURA et al.	)	Art Unit:
	)	Examiner:
I.A. No.: PCT/JP99/06474	)	Washington, D.C.
I.A. Filed: 19 November 1999	)	May 17, 2001
For: PROTEIN EXPRESSSION…	)	

PRELIMINARY AMENDMENT

Honorable Commissioner for Patents
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and prior to calculation of the filing fee, kindly amend as follows:

IN THE SPECIFICATION

After the title please insert the following paragraph:

REFERENCE TO RELATED APPLICATIONS

--The present application is the national stage under 35 U.S.C. §371 of international application PCT/JP99/06474, filed 19 November 1999 which designated the United States, and which application was not published in the English language.--

REMARKS

The above amendment to the specification is being made to insert reference to the PCT application of which the present case is a U.S. national stage.

Favorable consideration is earnestly solicited.

Respectfully submitted,
BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant

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PROTEIN EXPRESSION VECTOR AND USE THEREOF

FIELD OF THE INVENTION

The present invention relates to a protein expression vector and use thereof. More particularly, it relates to a protein expression vector which can express a gene encoding a target protein in various hosts to produce said protein. The present invention is advantageous and characterized technically in that a target protein can be expressed in a state of a recombinant fusion protein that is easy to be purified and is secreted extracellularly as well as in that a target protein can be obtained eventually in a state where the N-terminus of the target protein is free of addition of any extra amino acid.

BACKGROUND OF THE INVENTION

A variety of expression vectors have heretofore been developed for using in the production of recombinant proteins. In particular, for the expression systems utilizing microorganisms such as *Escherichia coli* and yeast as hosts, there have been provided those which are expected to give high yields. In the case of proteins whose biological activity depends on sugar chains, it is necessary to produce such proteins by using animal cells as

the host. In this regard, recently, a vector which permits a high level expression has also been developed (JP 10-179169 A), and there is an example of successful expression of human mannan binding protein by using this vector.

Thus, systems utilizing *Escherichia coli*, yeast or animal cells have been used by many investigators in order to produce foreign proteins. In the systems utilizing *Escherichia coli* as the host, expressing capacity can be enhanced by using a potent promoter derived from *Escherichia coli*. However, in most cases, foreign proteins expressed accumulate within cells as inclusion bodies. Therefore, it is necessary to solubilize the protein by using a denaturing agent such as urea and guanidine and then to unwind the protein to the native form. Then, it is extremely difficult to directly isolate and purify the protein in the active form, and complicated procedures are required.

Further, in the system utilizing yeast as the host, a proteolytic degradation is unavoidable. Then, improvement in the expression of soluble proteins can not be expected. In addition, the proteins are modified in a different way because of remarkably different expressing environment from the intercellular environment of higher animals. Furthermore, although systems utilizing animal cells may allow the production of recombinant proteins in

forms comparable to natural ones, complicated procedures are needed, thereby having a drawback with respect to production efficiency.

In recent years, an expression system has received an attention, wherein insect cells are used as the host infected with a baculovirus. The reason for this is, for example, that the baculovirus, upon infecting insect cells, produces more than approximately 25% of the total cell protein as a polyhedron protein, and a high expression system for foreign proteins has been developed by using this potent promoter. And, the following advantages have been recognized in regard to the production of foreign proteins by using a baculovirus-insect cell expression system: (a) the expression levels of foreign proteins are high; (b) processing of signal peptides, modification with sugar chains, phosphate, lipids, etc., dimerization, virion formation, intron splicing, and the like take place as those in natural proteins; (c) the intracellular localization of protein within insect cells is the same as that with the natural protein; (d) insect cells can be cultivated in a suspension culture.

Heretofore, a variety of proteins (e.g., insulin, interferons, erythropoietin, mannan binding protein, conglutinin, etc.) have been produced in insect cells and animal cells by using gene engineering technology. In

order to obtain recombinant proteins with quality comparable to that of the natural form, an expression system utilizing animal cells (e.g., mammalian cells or insect cells) as hosts is essential as described above. Then, the development of expression vectors which are useful in said expression system has been desired.

The development of expression vectors has been attempted primarily along two approaches, namely an attempt to enhance the expression level of recombinant proteins, and an attempt to simplify the purification of expressed recombinant proteins. Vectors which aim at enhancing the expression level include, for example, the vector disclosed in JP 10-179169 A. As vectors which aim at enhancing the purification efficiency, histidine Tag vector (manufactured by Invitrogen Corporation) is known.

pSecTag vector (manufactured by Invitrogen Corporation) is commercially available as a vector which facilitates purification of recombinant proteins secreted extracellularly. This vector is used with animal cells as the host, and contains a secretory signal, a multicloning site capable of inserting a nucleotide sequence encoding a target protein, a myc epitope which recognizes a fusion protein, and a polyhistidine Tag which allows purification of the protein by a nickel chelate resin. However, this vector can not express a target protein in insect cells.

Also, even if a protein is expressed in animal cells, amino acids such as myc epitope and histidine Tag are added to the C-terminus of a target protein, precluding the protein from being obtained as a pure recombinant protein, which is a drawback of using this vector.

On the other hand, pFastBAC HT vector (manufactured by GIBCO BRL) is commercially available as a vector which enables proteins to be expressed in insect cells and to be purified easily. This vector uses insect cells as the host and contains a histidine Tag nucleotide sequence, a cleavable nucleotide sequence which allows the cleavage of the sequence between that encoding the histidine Tag sequence and that encoding a target protein, and a multicloning site capable of inserting the nucleotide sequence encoding the target protein. However, this vector does not contain a secretory signal which enables extracellular secretion of a target protein to. Therefore, cells must be disrupted in order to obtain a target protein expressed intracellularly. A myriad of proteins within the cells will be released by cell disruption, making it extremely difficult to purify the target protein.

Also, it is desirable that an expressible recombinant protein is identical to the corresponding natural protein in its amino acid sequence, with no expression vector-derived amino acids being added to the C-

terminus or the N-terminus. In particular, it has been known that the type of the amino acid at position 1 (N-terminus) of a natural or recombinant protein markedly affects the stability of said protein. That is, there is a strong correlation between the property of the N-terminal amino acid and the in vivo half life of the protein, which is designated as the N-end rule. This correlation holds true to a greater or lesser extent with proteins of every living system that has been so far studied spanning from bacteria to mammals.

Under the above-described circumstances, it has been desired to develop an expression vector that can express recombinant proteins in an expression system which can utilize animal cells, mammalian cells or insect cells in particular, as the host and can secrete the protein extracellularly, wherein the obtained recombinant can be purified by a simple procedure, and still further at least the N-terminus of the amino acid sequence of the recombinant protein is identical to that of the natural protein.

OBJECTS OF THE INVENTION

Accordingly, the primary object of the present invention is to provide a novel expression vector which can express recombinant proteins in various hosts such as

animal cells, particularly, mammalian cells or insect cells, and can secrete the proteins extracellularly, wherein the obtained recombinant can be purified by a simple procedure, and still further at least the N-terminus of the amino acid sequence of the recombinant protein is identical to that of the natural protein.

SUMMARY OF THE INVENTION

The present invention provides an expression vector which, upon using in various host cells (particularly animal cells such as mammalian cells and insect cells), can secrete a recombinant protein produced extracellularly, allows the simple purification of the produced recombinant protein, and still further provides the recombinant protein almost identical in quality to the natural protein. The expression vector provided herein may also be used in situations where to use microorganisms and the like as the host is preferred, for example, where the presence of sugar chains on the protein is not necessary, or protein production is carried out as a basic study.

The protein expression vector of the present invention contains as the basic construction at least (1) a nucleotide sequence for a secretory signal and, in the 3′ downstream side thereof, (2) a nucleotide sequence for Tag, (3) a cleavable nucleotide sequence, and (4) a nucleotide

sequence encoding a target protein or (4′) a cloning site into which a target protein-encoding nucleotide sequence can be inserted, in this order. The vector may also contain, as appropriate, an optional nucleotide sequence such as a nucleotide sequence encoding an epitope or a nucleotide sequence encoding a spacer sequence before, after or between the essential nucleotide sequences of (1) through (4) or (4′).

That is, according to the present invention, there is provided:

(1) A protein expression vector comprising a secretory nucleotide signal and, in the 3′ downstream side thereof, a Tag nucleotide sequence, a cleavable nucleotide sequence and a cloning site into which a nucleotide sequence encoding a target protein can be inserted, in this order;

(2) The protein expression vector according to the above (1), wherein a nucleotide sequence encoding a target protein is inserted in the cloning site;

(3) The protein expression vector according to the above (1) or (2), wherein the cloning site or the nucleotide sequence encoding the target protein is present successively at the 3′ end of the cleavable nucleotide sequence;

(4) The protein expression vector according to

any one of the above (1) to (3), wherein a nucleotide sequence encoding at least on amino acid is contained as a spacer nucleotide sequence in the 3′ downstream side of the secretory signal nucleotide sequence, but in the 5′ upstream side of the cleavable nucleotide sequence;

(5) The protein expression vector according to the above (4), wherein the spacer nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Leu-Val-His-Gly-Lys-Leu;

(6) The protein expression vector according to the above (4) or (5), wherein the spacer nucleotide sequence is composed of at least a cleavable nucleotide sequence;

(7) The protein expression vector according to any one of the above (1) to (6), wherein the cleavable nucleotide sequence, when translated into an amino acid sequence, is cleaved by an enzyme at immediate upstream and/or immediate downstream and/or in the middle of said amino acid sequence;

(8) The protein expression vector according to the above (7), wherein the cleavable nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Asp-Asp-Asp-Asp-Lys;

(9) The protein expression vector according to the above (7) or (8), wherein the enzyme is enterokinase;

(10) The protein expression vector according to any one of the above (1) to (9), wherein the secretory signal nucleotide sequence is IgG (κ) signal or trypsin signal;

(11) The protein expression vector according to any one of the above (1) to (10), wherein the Tag nucleotide sequence is polyhistidine;

(12) The protein expression vector according to any one of the above (1) to (11) further comprising a nucleotide sequence encoding an antibody recognition epitope;

(13) The protein expression vector according to any one of the above (1) to (12), wherein the nucleotide sequence encoding the target protein is that encoding neurosin;

(14) Host cells transformed with the protein expression vector according to any one of the above (1) to (13);

(15). The host cells according to the above (14) which are animal cells;

(16) The host cells according to the above (15), wherein the animal cells are mammalian cells;

(17) The host cells according to the above (15), wherein the animal cells are insect cells;

(18) A process for producing a target protein

which comprises using the protein expression vector or the host cells according to any one of the above (1) to (18);

(19) A target protein which is obtained by the process according to the above (18);

(20) A process for producing a recombinant fusion protein comprising an amino acid sequence of a target protein which comprises using the protein expression vector or the host cells according to any one of the above (1) to (18);

(21) A recombinant fusion protein comprising the amino acid sequence of the target protein obtained by the process according to the above (20);

(22) A process for producing a target protein which comprises retaining the recombinant fusion protein according to the above (21) with a substance capable of recognizing Tag and/or an epitope in said recombinant fusion protein, liberating the recombinant fusion protein from the substance to purify it, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant fusion protein, followed by collecting the released target protein;

(23) A process for producing a target protein, which comprises retaining the recombinant fusion protein according to the above (21) with a substance capable of

recognizing Tag and/or an epitope in said recombinant fusion protein, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant protein, followed by collecting the released target protein; and

(24) A target protein is obtained by the process according to the above (22) or (23).

BRIEF DESCRIPTION OF THE DRAWINS

Fig. 1 illustrates construction of the plasmid pTrypHis/Neurosin produced by the process of Example 1.

Fig. 2 illustrates the western blot analysis of the culture supernatant and the cell extract obtained in Example 1.

Fig. 3 illustrates construction of the plasmids pSecTag/Neurosin, pSecHisTag/Neurosin, and pSecTrypHis/Neurosin of Example 2.

Fig. 4 illustrates the western blot analysis of the culture supernatant obtained in Example 2.

Fig. 5 illustrates construction of the plasmid pFBTrypSigTag/Neurosin obtained by the process of Example 3.

Fig. 6 illustrates the western blot analysis of the culture supernatant obtained in Example 3.

Fig. 7 illustrates a gel electrophoretic pattern

of recombinant human neurosin purified by a nickel column.

Fig. 8 illustrates the enzymatic activity of human neurosin expressed by using the baculovirus expression system.

DETAILED DESCRIPTION OF THE INVENTION

The term "host cells" as used herein refers to cells, irrespective of the type, which express a nucleotide sequence encoding a target protein within the protein expression vector of the present invention and secrete the protein extracellularly. Therefore, the host cells may be microorganisms, preferably animal cells, and most preferably mammalian or insect cells.

Specific examples of mammalian cells and insect cells include human-derived cells, mouse-derived cells, fly-derived cells, silk worm-derived cells, and the like. In particular, the cells to be used are selected from the group consisting of CHO cells, COS cells, BHK cells, Vero cells, myeloma cells, HEK293 cells, HeLa cells, Jurkat cells, mouse L cells, mouse C127 cells, mouse FM3A cells, mouse fibroblast cells, osteoblasts, chondrocytes, S2 cells, Sf9 cells, Sf21 cells, High Five® cells, and the like. Also, microorganisms such as *Escherichia coli* and yeast may be used.

The "protein expression vector" of the present

invention is preferably a vector which expresses a target protein as a recombinant fusion protein to facilitate isolation, purification or recognition. The term "recombinant fusion protein" refers to a protein, wherein an appropriate protein is attached to the N-terminus and/or the C-terminus of a target protein. In this connection, the term "recombinant protein" is also used herein, and this refers to a recombinant fusion protein produced by integrating a nucleotide sequence encoding a target protein into the protein vector of the present invention and expressing the fusion protein from which an amino acid sequence derived from other than the nucleotide encoding the target protein is deleted by cleavage. Then, it is substantially a synonym of a target protein.

The protein expressed by the protein expression vector of the present invention and secreted extracellularly is a fusion protein comprising at least a target protein, a Tag sequence, and an amino acid sequence containing a cleavable site between the Tag sequence and the target protein. In addition, said fusion protein may further contain an epitope that can be recognized by an antibody, or the Tag sequence may function as an epitope. The desired recombinant protein can be obtained by subjecting the thus-expressed recombinant protein to an appropriate processing.

After translation, an active protein may be obtained. Even when the resultant protein is not an active protein, it may be converted to an active protein by applying a variety of processing. In many cases, a protein is first synthesized at the ribosomes in the cytoplasm as an inactive precursor (pro-form) which comprises an active protein bearing at the N-terminus thereof a peptide of about 15 to 60 amino acids responsible for secretion (secretory signal). The peptide region, which functions as a secretory signal, is concerned with the mechanism of passing through the cell membrane, and is removed by cleavage with a specific protease during the passage through the membrane (not always) to yield a mature protein. The peptide moiety which functions as a secretory signal has a broad hydrophobic region comprising hydrophobic amino acids in the middle of the sequence, and basic amino acid residues at a site close to the N-terminus. A secretory signal may be understood as a synonym of a signal peptide.

In addition, in some proteins, a peptide moiety which functions as a secretory signal is further attached to the N-terminus of an inactive precursor (pro-form), and such a protein is called as a prepro-protein (the prepro-form). For example, trypsin is present as a prepro-form immediately after translation into amino acids, as a pro-form after being secreted from cells, and is converted into

active trypsin in duodenum upon limited degradation by enteropeptidase or by self degradation. A pro-form from which an active protein region has been deleted is called as a pro-region, a prepro-form from which a pro-form region has been deleted is called as a pre-region, and a prepro-form from which an active protein region has been deleted is called as a prepro-region.

The "secretory signal nucleotide sequence", which is one of the essential components of the protein expression vector of the present invention, refers to the nucleotide sequence encoding a secretory signal. Also, the "secretory signal" refers to the pro-region when a protein expressed as a pro-form, and at least the pre-region or the prepro-region when a protein expressed as a prepro-form. However, the secretory signal is not limited in so far as it is capable of secreting the intracellularly expressed protein, extracellularly. The secretory signal nucleotide sequence constructed within the protein expression vector of the present invention preferably encodes a secretory signal with a cleavage site at the C-terminus of the signal. When the sequence encodes a secretory signal that does not contain a cleavage site at the C-terminus, it is preferred to newly insert a nucleotide sequence encoding a cleavable site at the 3′ end of said secretory signal nucleotide sequence. This is, for example, a trypsin signal

represented by 1st to 23rd amino acids in SEQ ID NO: 19. At the C-terminus (19th to 23rd amino acids) of said sequence, there is Asp-Asp-Asp-Asp-Lys which is recognizable by enterokinase.

Since the secretory signals of eukaryotic cells are similar to those of prokaryotic cells, *Escherichia coli* and the like may be used as the host. Since the secretory signal has different extracellular secretory activities depending on the host, it is necessary to select a secretory signal appropriate to the host. Specific examples of secretory signals include IgG (κ) (or IgGk) signal (or leader) and trypsin signal, which exhibit particularly high secretory activities when insect cells or mammalian cells are used as the host cells. Other examples of secretory signals include BiP of flies (*Drosophila*), melitin of honeybees, α-factor of Pichia pastoris, PHO, and the like. When trypsin signal is referred herein, it may be constructed by either 1st to 18th amino acids or 1st to 23rd amino acids in SEQ ID NO: 19. Further, the secretory signal also includes, other than those exemplified above, their homologs and variants which are capable of secreting proteins extracellularly.

The "Tag nucleotide sequence", which is another essential component of the protein expression vector of the present invention, refers to a nucleotide sequence that

encodes Tag sequence. The "Tag sequence" refers to an amino acid sequence that is no derived from the nucleic acid encoding a target protein and is inserted in order to facilitate, when expressed, isolation, purification and recognition of the target protein. Therefore, such a Tag sequence may be, for example, an antigen or an epitope recognizable by an antibody. By retaining the recombinant fusion protein containing a Tag sequence in a substance capable of recognizing said Tag sequence, isolation and purification can be carried out easily.

As a specific example of the isolation and purification process, the recombinant protein may be isolated and purified by retaining the recombinant fusion protein obtained by the present invention in a substance capable of recognizing, for example, Tag sequence, followed by liberating the fusion protein to obtain the recombinant fusion protein, which is further reacted with an enzyme capable of recognizing and cleaving the cleavable sequence. The recombinant protein may also be isolated and purified by reacting the recombinant fusion protein of the present invention, while it is retained by a substance capable of recognizing Tag sequence, with an enzyme capable of recognizing and cleaving the cleavable sequence, without undergoing the liberation process.

Specific examples of Tag nucleotide sequences

include a nucleotide sequence which encodes polyhistidine (PHIS; hereinafter also referred to as histidine Tag or His tag) comprising preferably six histidines ((His)6). The recombinant fusion protein, which is obtained by expressing the PHIS-encoding nucleotide sequence using the protein expression vector of the present invention, contains PHIS as the Tag sequence. PHIS is absorbed, for example, by a nickel-chelating resin (ProBond®), which can be desorbed from said resin by pH variation or by adding EDTA or an imidazole substance. The recombinant fusion protein can be isolated and purified by utilizing such properties.

In another example, glutathione-S-transferase (GST) is used as a Tag sequence, wherein affinity chromatography is run by using a glutathione Sepharose 4B column capable of recognizing GST, after which the recombinant protein can be isolated and purified by adding glutathione to allow competitive binding.

In still another example, calmodulin binding peptide (CBP) may be used as a Tag sequence, wherein affinity chromatography is run by using a calmodulin affinity resin capable of recognizing CBP, after which the recombinant protein can be isolated and purified by the addition of EGTA.

In still another example, protein A is used as a Tag sequence, wherein affinity chromatography is run by

using an IgG Sepharose 6FF column capable of recognizing protein A, after which the recombinant protein can be isolated and purified by a treatment such as pH variation.

The "cleavable nucleotide sequence", which is still another essential component of the protein expression vector of the present invention, refers to a nucleotide sequence, wherein after said nucleotide acid sequence is translated into the amino acid sequence, said amino acid sequence can be cleaved at immediate upstream and/or immediate downstream and/or in the middle thereof.

For example, a nucleotide sequence encoding an amino acid sequence which is susceptible to enzyme-specific cleavage corresponds to this sequence. Examples thereof include as follows: a nucleotide sequence encoding the amino acid sequence of Asp-Asp-Asp-Asp-Lys (said amino acid sequence is recognized by enterokinase, and the recombinant fusion protein is cleaved at the C-terminus thereof); a nucleotide sequence encoding the amino acid sequence of Leu-Val-Pro-Arg-Gly-Ser (said amino acid sequence is recognized by thrombin, and the recombinant fusion protein is cleaved between Arg-Gly thereof); a nucleotide sequence encoding the amino acid sequence Ile-Glu-Gly-Arg (said amino acid sequence is recognized by factor Xa, and the recombinant fusion protein is cleaved at the C-terminus thereof); a nucleotide sequence encoding the amino acid

sequence Glu-Asn-Leu-Tyr-Phe-Gln (said amino acid sequence is recognized by TEV (Tobacco Etch virus) protease, and the recombinant fusion protein is cleaved at the C-terminus thereof), and the like.

The cleavable nucleotide sequence may be constructed by utilizing a part or all of the nucleotide sequence encoding the secretory signal nucleotide sequence, the Tag nucleotide sequence or the target protein, with or without an appropriate nucleotide sequence being added to it.

The protein expression vector of the present invention contains, in addition to the above-described three essential components, a nucleotide sequence encoding a target protein or a cloning site into which said nucleotide can be inserted, in the 3′ downstream side of the essential components. The nucleotide sequence encoding the target protein is not specifically limited and a nucleotide sequence encoding insulin, interferons, erythropoietin, mannan binding protein, conglutinin, neurosin, or the like may be used.

Any backbone vector may be used for the protein expression vector of the present invention as far as the above essential components are present, but it is desirable to use one which fits to the host cells. A backbone vector refers to a vector that is used as a source material such

as pSecTag2A, pSecTag2B, pFastBAC1, or the like as described in the Examples. The backbone vector is not specifically limited as far as it is a vector capable of expressing proteins, examples of which include pBAD/His, pRSETA, pcDNA2.1, pTrcHis2A, pYES2, pBlueBac4.5, pcDNA3.1 and pSecTag2 manufactured by Invitrogen Corporation, pET and pBAC manufactured by Novagen Company, pGEM manufactured by Promega Biotec, pBluescript II manufactured by Stratagene Company, pGEX and pUC18/19 manufactured by Pharmacia Corporation, pRTE, pEBFP and pGAD GH manufactured by Clontech Company, and the like.

Furthermore, a promoter and/or enhancer may be derived from the backbone vector, or they may be replaced, added or deleted to fit the host as appropriate. Promoters or enhancers which may be used include, for example, T7, CMV, HSV TK, SV40, RSV, trc, BAD, TRE-minCMV, 5' LTR, GAL 1, AOX 1, lac, ADH 1, polyhedrin, metallothionein, actin 5C gene, and the like.

The protein expression vector of the present invention may further include, in addition to the above essential components, a "spacer nucleotide sequence". A spacer nucleotide sequence refers to a nucleotide sequence encoding a spacer sequence, and may be inserted at any site within the protein expression vector of the present invention. A spacer sequence is an amino acid sequence

(usually composed of about 1 to 50 amino acids) which is different from any of the secretory signal, the Tag sequence, the epitope sequence and the target protein, and plays a role as an auxiliary mean capable of secreting the target protein as a result.

A space sequence may be, for example, a cleavable sequence from which the secretory signal, the Tag sequence and epitope can be cleaved by enzyme, or the like. In particular, in the case where there is a histidine Tag upstream of the target protein, inserting successively a prepro-region in the secretory signal and inserting the amino acid sequence Leu-Val-His-Gly-Lys-Leu as a spacer sequence to the C-terminus of the prepro-region are convenient for the cleavage by an enzyme, or the like, because the distance between the trypsin signal and the histidine Tag becomes larger.

The protein expression vector of the present invention may also contain a nucleotide sequence encoding an "antibody recognition epitope". An antibody recognition epitope refers to an antigen determinant that is recognized by the antibody and is a region which is capable of binding to the antibody. The antibody may be any of monoclonal antibody, polyclonal antibody, antiserum, and the like. In the case where an epitope is expressed in such a way that it is contained in the recombinant fusion protein, the

expression of the recombinant fusion protein can be confirmed by using an antibody against said epitope, and the protein is isolated and purified easily by an antigen-antibody affinity column, and further the recombinant protein can be obtained by cleaving the protein at the cleavable site as needed. Examples of expressible epitopes include Xpress, thioredoxin, c-myc, V5, HA/c-myc, and the like.

Introduction of the above expression vectors into the host cells per se may be conducted by employing one of conventional methods which include, for example, transfection by the lipopolyamine method, the DEAE-dextran method, Hanahan's method, the lipofectin method, and the calcium phosphate method, microinjection, electroporation, and the like.

The present invention includes, in addition to the protein expression vector of the above composition, host cells that are transformed by said protein expression vector, the process for production of the recombinant fusion protein that expresses the recombinant fusion protein by cultivating said transformed host cells, the recombinant fusion protein obtained by the process of said production process, the process for production of the recombinant protein wherein the recombinant protein is produced from said recombinant fusion protein, and the

recombinant protein obtained by said production method.

EXAMPLES

The following Examples further illustrate the present invention in detail but are not to be construed to limit the scope of the present invention. In the following Examples, IgGk leader may be understood as a synonym of the secretory signal of IgG. When DDDDK (Asp-Asp-Asp-Asp-Lys) is present proximate to a trypsin signal, the DDDDK and the trypsin signal inclusive is called as trypsin signal in some cases (the sequence of 1st to 23rd amino acids in SEQ ID NO: 19), whereas only the trypsin signal without containing said DDDDK is as called trypsin signal (the sequence of 1st to 18th in SEQ ID NO: 19) in other cases. Those skilled in the art can readily understand that a particular sequence corresponds to either of which from the context of the description. The trypsin signal shown in Figs. 1, 3 and 5 refers to the 1st to 18th amino acids in SEQ ID NO: 19. In this connection, IgGk signal and the trypsin signal may be used in an interchangeable manner and, in this resepct, both are considered to be equivalent, and the trypsin signal referred to herein may or may not include DDDDK.

Example 1

Construction and expression of plasmid

pTrypTag/Neurosin

A sense DNA containing the nucleotide sequence shown in SEQ ID NO: 1 and an antisense DNA containing the nucleotide sequence shown in SEQ ID N: 2 were synthesized as a secretory signal containing a histidine Tag (His tag) (hereinafter referred to as His secretory signal) to be newly incorporated into the plasmid pSecTag2A (manufactured by Invitrogen Corporation). The sequences of the restriction site in this His secretory signal sequence were Hind III-Nhe I at the 5′ end and BamH I-EcoR I at the 3′ end.

Plasmid pSecTag2A (1 μg, 0.1 μl) was treated with the restriction enzymes Nhe I and BamH I to completely remove the region encoding IgGk leader sequence. To this solution were added 100 pmoles each of the sense DNA and the antisense DNA described above, and the mixture was treated at 70°C for 10 minutes, after which it was left standing at room temperature for 30 minutes to allow annealing. To 1 μl each of the His secretory signal sequence, which had been treated with Nhe I and BamH I, and pSecTag2A was added 2.0 μl of solution I of DNA Ligation Kit Ver. 2 (Takara Shuzo Co., Ltd.), and the mixture was allowed to react at 16°C for 30 minutes. To the reaction mixture was added 0.1 ml of competent *Escherichia coli* cells XL1-Blue (Stratagene Company), and the mixture was

allowed to react on ice for 30 minutes, followed by heat shock at 42°C for 60 seconds. After the reaction mixture was left on ice for 2 minutes, 0.9 ml of the SOC medium (Toyobo Co., Ltd.) was added and the cells were shake-cultured at 37°C for one hour. The culture was centrifuged at 5,000 rpm for one minute and the supernatant was discarded. The sedimented competent cells was suspended in the solution remaining in the centrifugation tube, and applied to two ampicillin LB plates containing 100 μg/ml ampicillin at a ratio of 1 : 10. The cells were cultivated overnight at 37°C and, from plasmids obtained from the resulting colonies, those with inserted DNA of the His secretory signal were selected by PCR and designated as pTrypHis.

pTrypHis was recovered by using a Pharmacia Flex Prep kit from *Escherichia coli* cells which were cultivated over day and night. To 5 μg of pTrypHis vector was added 20 units of BamH I and the vector was cleaved at 37°C for 4 hours, after which 6 units of mung-bean exonuclease (Takara Shuzo Co., Ltd.) was added. The mixture was allowed to react at room temperature (25°C) for 30 minutes to blunt the ends. Further, the 3′ end of the cloning site was cleaved with 20 units of EcoR I, after which one unit of bacterial alkaline phosphatase (Takara Shuzo Co., Ltd.) was added. The mixture was reacted at 65°C for 30 minutes.

The inserted human neurosin cDNA was subjected to amplification by PCR by using the cDNA, which had already been cloned into pSPORT 1 (Gibco BRL), as the templates, at a portion corresponding to SEQ ID NOS: 3 and 4. In this case, the 5′ end of SEQ ID NO: 3 was phosphorylated in advance by T4 polynucleotide kinase (Takara Shuzo, Co., Ltd.).

The thus-obtained PCR product was precipitated once by ethanol, after which the 3′ end was cleaved by EcoR I. This cDNA and the above-mentioned pTrypHis were separated by electrophoresis on 1.0% agarose, and the target bands were cut out and purified by Sephaglas BandPrep kit (Pharmacia Corporation). They were then ligated in the same manner as described above and introduced into *Escherichia coli* XL1-Blue. Clones containing the sequence for neurosin were selected as pTRypHis/Neurosin (Fig. 1), and the plasmid DNA was recovered. One microgram of pTrypHis/Neurosin (1 μg) was introduced into COS-1 cells by using LipofectAMINE (Gibco BRL) according to the instruction manual. At 48 to 72 hours after introduction, the culture supernatant and the cell extract were recovered and subjected to western blot analysis using an anti-neurosin antibody (JP 10-187506 A) according to a conventional method, results of which demonstrated that the recombinant neurosin was present only

in the cell extract (Fig. 2).

The nucleotide sequence and the amino acid sequence of human active-form neurosin are shown in SEQ ID NOS: 14 and 15.

Example 2

Studies on preparation and expression of pSecTag/Neurosin, pSecHisTag/Neurosin, and pSecTrypHis/Neurosin

(1) Construction of each plasmid

According to the same manner as in Example 1, cDNA corresponding to the active region of neurosin, which was amplified by SEQ ID NOS: 5 and 6 and using as the template pTrypHis/Neurosin, was inserted between Hind III site and Xho I site of pSecTag2B cloning site to obtain pSecTag/Neurosin (Fig. 3A). cDNA was amplified by using SEQ ID NOS: 7 and 4 and as the template pTrypHis/Neurosin constructed in Example 1, and was inserted between Hind III and EcoR I sites of pSecTag2B to obtain pSecHisTag/Neurosin (Fig. 3B). According to the same manner as in Example 1, SEQ ID NOS: 8 and 9 were annealed, and the fragment obtained by Nhe I and BamH I digestion was inserted into pSecTag2A to obtain pSecTrypHis. Into BamH I site and Xho I site of pSecTrypHis, which had been blunt-ended, was inserted active-form neurosin which had been amplified by SEQ ID NOS: 3 and 6 according to the same manner as in

Example 1, to obtain pSecTrypHis/Neurosin (Fig. 3C).

The nucleotide sequence and amino acid sequence in upstream of cDNA of the region of active human neurosin in Fig. 3B, i.e, the region IgGk leader-spacer sequence-(His)6-DDDDK, are shown in SEQ ID NOS: 16 and 17. The IgGk leader corresponds to the 1st to 21st amino acids, the spacer sequence corresponds to the 22nd to 34th amino acids, (His)6 corresponds to the 35th to 40th amino acids, and DDDDK corresponds to the 41st to 45th amino acids.

(2) Expression of each plasmid in COS-1 cells

Each plasmid DNA (1 μg) was introduced into COS-1 cells according to the same method as in Example 1, and the cell extract and culture supernatant obtained after 48 to 72 hours were subjected to western blot analysis for the presence of recombinant neurosin protein by using an anti-neurosin antibody. The results demonstrated that neurosin was secreted into the culture supernatant in all of the supernatants studied, and that neurosin was secreted when at least the signal peptide and several amino acids at the C-terminus thereof were present. Also, there was no difference in secreting efficiency observed between the cases where the signal sequences of IgGk and trypsionogen are used (Fig. 4).

Example 3

Preparation of pFBTrypSigTag/Neurosin

The portion of pSecTrypHis/Neurosin spanning from the trypsin signal to the enterokinase recognition site was amplified by using SEQ ID NOS: 10 and 11 such that the peptide Leu-Val-His-Gly was located at the C-terminus. The product was inserted between Nhe I and Hind III sites of pSecTag2A to obtain the plasmid pTrypSig. About 200 bp which contained His tag region in pTrypHis was amplified by using SEQ ID NOS: 11 and 7. A fragment of about 40 bp containing His tag and enterokinase recognition site, which was produced by digesting with Hind III and BamH I, was inserted into pTrypSig to obtain pTrypSigTag (Fig. 5A).

CDNA, prepared by amplification of the portion from the trypsin signal sequence to the enterokinase recognition site of pTrypSigTag by PCR using SEQ ID NOS: 6 and 12, was cleaved out by Bg III and BamH I digestion, and inserted into BamH I site of pFastBac 1 (manufactured by Gibco Company). The direction of the insertion was confirmed by PCR using SEQ ID NOS: 6 and 13, and clones with the sequence inserted in the direction to be transcribed and translated by polyhedrin promoter were selected to obtain pFBTrypSigTag. To this was inserted the active form of neurosin according to the same manner as in Example 1 to obtain pFBTrypSigTag/Neurosin (Fig. 5 B). In this case, the nucleotide sequence was determined by using a fluorescence-labeled SEQ ID NO: 10 to check whether or

not neurosin was inserted correctly.

The nucleotide sequences and amino acid sequence of upstream of cDNA for the human active neurosin region in Fig. 5B, i.e., the nucleotide sequence and amino acid sequence of the region trypsin signal-DDDDK-spacer sequence-(His)6 - DDDDK, are shown in SEQ ID NOS: 18 and 19. The trypsin signal-DDDDK corresponds to the 1st to 23rd amino acids, the spacer sequence corresponds to the 24th to 29th amino acids, (His)6 corresponds to 30th to 35th amino acids, and the succeeding DDDDK corresponds to 36th to 40th amino acids.

pFBTrypSigTag/Neurosin was processed according to the protocol of the Gibco BRL BAC-TO-BAC baculovirus expression system to obtain a recombinant bacmid containing on the bacmid DNA a chimeric neurosin fused with the trypsinogen signal peptide, the His tag, and the enterokinase recognition site. When this bacmid was expressed in Sf-9 cells according to the manual of the BAC-TO-BAC baculovirus expression system, it was demonstrated by western blotting using an anti-neurosin antibody that neurosin was secreted in the culture supernatant from day 2 after viral infection (Fig. 6).

Western blotting may be carried out according to the following method. That is, after the culture supernatant was recovered, it was mixed with an equal

volume of 2 x SDS loading buffer (manufactured by Daiichi Pure Chemicals Co., Ltd.), and the mixture was heated in a boiling bath for 5 minutes to prepare a sample solution. The sample solution was subjected to electrophoresis on 10 to 20% polyacrylamide gel (manufactured by Daiichi Pure Chemicals Co., Ltd.) using an SDS electrophoretic apparatus (manufactured by Daiichi Pure Chemicals Co., Ltd.) and a SDS-tris-glycine buffer (manufactured by Daiichi Pure Chemicals Co., Ltd.). During the electrophoresis, two sheets of 3MM filter paper (manufactured by Whatman Company) were immersed in the anolyte 1 (manufactured by Daiichi Pure Chemicals Co., Ltd.), one sheet in anolyte 2 (manufactured by Daiichi Pure Chemicals Co., Ltd.) and three sheets in a catholyte (manufactured by Daiichi Pure Chemicals Co., Ltd.). Also, a polyvinylidene difluoride membrane (PVDF membrane: manufactured by Millipore Corporation) was immersed in methanol and then in distilled water to make it non-water repelling.

For the transfer of the proteins to the PVDF membrane, the gel was removed from the apparatus after the electrophoresis, and then on a blotter (manufactured by Pharmacia Company) were placed two sheets of filter paper immersed in buffer A from the anode, one sheet of filter paper immersed in buffer B, the PVDF membrane, the gel, and three sheets of filter paper immersed in buffer C in the

order of description, whereby carrying out the transfer at 8 mV/cm^2 for 1.5 hours. After the transfer, the PVDF membrane was blocked by shaking in BlockAce (manufactured by Snow Brand Milk Products Co., Ltd.). Thereupon, said membrane was reacted overnight at 4°C with an anti-neurosin antibody diluted with PBS containing 5% fetal bovine serum. Thereafter, alkaline phosphatase-labeled mouse IgG antibody was added and, after the reaction at room temperature for one hour, the color was developed with a NBT-BCIP solution to confirm the expression of the recombinant neurosin protein in the culture supernatant (Fig. 6).

Further, the recombinant fusion protein (neurosin) obtained in the culture supernatant was purified by passing through a chelate column, and assayed for the enzyme activity after dialysis. First, the culture supernatant was subjected to a chelate column (Ni-NTA-Agarose, manufactured by Qiagen Company) by using the PBS buffer, and eluted in a stepwise manner (5, 10, 100, 500 mM) with solutions of imidazole dissolved in PBS (manufactured by Wako Pure Chemical Industries, Ltd.). Each fraction was subjected to electrophoresis and confirmed by the western blotting method and the Coomassie staining (Fig. 7). The western blotting was carried out according to the above described method, and Coomassie staining was carried out by immersing the electrophoresis

gel in a solution of Coomassie brilliant blue for 10 minutes. Thereupon, the gel was destained in a destaining solution (water: acetic acid: methanol = 33: 6: 21).

The fraction obtained by eluting with 100 mM of imidazole was further replaced by the PBS buffer in a PD-10 column (manufactured by Pharmacia Company). Ten microliter of enterokinase (1 U/μl, manufactured by Invitrogen Corporation) was mixed with 50 μl of this sample, and the mixture was reacted at room temperature for 60 minutes. Next, 50 μl of a 0.2 M solution of a substrate, which was prepared by dissolving a synthetic substrate of Boc-Gln-Ala-Arg-MCA (Peptide Institute) in DMSO and by diluting in 1 M Tris-HCl (pH 8.0), was added and the mixture was reacted at 37°C. Fluorescence at an excitation wavelength of 380 nm and a fluorescence emission wavelength of 460 nm was determined sequentially (after 1, 2, 4, 5, and 15 hours) (Fig. 8). The values shown in the figure are those obtained after subtracting the fluorescence value of EK only.

INDUSTRIAL UTILITY

The protein expression vector of the present invention is advantageous and characterized by in that the protein expression vector has the above-described specific construction of the components thereby facilitating the

purification and recovery of a target protein in a mature form or an active form. A preferred example of the construction of said protein expression vector includes a secretory signal nucleotide sequence, a Tag nucleotide sequence positioned in the 3' downstream thereof, a cleavable nucleotide sequence comprising a nucleotide sequence encoding the amino acid sequence of Asp-Asp-Asp-Asp-Lys capable of being recognized by enterokinase, a nucleotide sequence encoding the target protein positioned successively in the downstream, and a nucleotide sequence containing a stop codon positioned in the furthest downstream, where it is possible by using this vector to produce a recombinant protein without additional amino acids attached to the N-terminus or the C-terminus of the target protein, namely the target protein of a mature form or an active form.

SEQUENCE LISTING FREE TEXT

SEQ ID NO: 1: Designed oligonucleotide to construct plasmid pTrpHis.

SEQ ID NO: 2: Designed oligonucleotide to construct plasmid pTrpHis.

SEQ ID NO: 3: Designed oligonucleotide primer to amplify neurosin-encoding sequence.

SEQ ID NO: 4: Designed oligonucleotide primer to

amplify neurosin-encoding sequence.

SEQ ID NO: 5: Designed oligonucleotide primer to amplify a portion of plasmid pTRypHis/Neurosin.

SEQ ID NO: 6: Designed oligonucleotide primer to amplify a portion of plasmid pTrypHis/Neurosin.

SEQ ID NO: 7: Designed oligonucleotide primer to amplify a portion of plasmid pTrypHis/Neurosin.

SEQ ID NO: 8: Designed oligonucleotide to construct plasmid pSecTrypHis.

SEQ ID NO: 9: Designed oligonucleotide to construct plasmid pSecTrypHis.

SEQ ID NO: 10: Designed oligonucleotide primer to amplify a portion of plasmid pSecTryp/Neurosin.

SEQ ID NO: 11: Designed oligonucleotide primer to amplify a portion of plasmid pSecTryp/Neurosin.

SEQ ID NO: 12: Designed oligonucleotide primer to amplify a portion of plasmid pTrypSigTag.

SEQ ID NO: 13: Designed oligonucleotide primer to amplify a portion of plasmid pFBTrypSigTag.

What is claimed is:

1. A protein expression vector comprising a secretory nucleotide signal and, in the 3′ downstream side thereof, a Tag nucleotide sequence, a cleavable nucleotide sequence and a cloning site into which a nucleotide sequence encoding a target protein can be inserted, in this order.

2. The protein expression vector according to claim 1, wherein a nucleotide sequence encoding a target protein is inserted in the cloning site.

3. The protein expression vector according to claim 1 or 2, wherein the cloning site or the nucleotide sequence encoding the target protein is present successively at the 3′ end of the cleavable nucleotide sequence.

4. The protein expression vector according to any one of claims 1 to 3, wherein a nucleotide sequence encoding at least on amino acid is contained as a spacer nucleotide sequence in the 3′ downstream side of the secretory signal nucleotide sequence, but in the 5′ upstream side of the cleavable nucleotide sequence.

5. The protein expression vector according to claim 4, wherein the spacer nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Leu-Val-His-Gly-Lys-Leu.

6. The protein expression vector according to claim 4 or 5, wherein the spacer nucleotide sequence is composed of at least a cleavable nucleotide sequence.

7. The protein expression vector according to any one of claims 1 to 6, wherein the cleavable nucleotide sequence, when translated into an amino acid sequence, is cleaved by an enzyme at immediate upstream and/or immediate downstream and/or in the middle of said amino acid sequence.

8. The protein expression vector according to claim 7, wherein the cleavable nucleotide sequence is a nucleotide sequence encoding at least the amino acid sequence of Asp-Asp-Asp-Asp-Lys.

9. The protein expression vector according to claim 7 or 8, wherein the enzyme is enterokinase.

10. The protein expression vector according to any one of claims 1 to 9, wherein the secretory signal nucleotide sequence is IgG (κ) signal or trypsin signal.

11. The protein expression vector according to any one of claims 1 to 10, wherein the Tag nucleotide sequence is polyhistidine.

12. The protein expression vector according to any one of claims 1 to 11 further comprising a nucleotide sequence encoding an antibody recognition epitope.

13. The protein expression vector according to any one of claims 1 to 12, wherein the nucleotide sequence

encoding the target protein is that encoding neurosin.

14. Host cells transformed with the protein expression vector according to any one of claims 1 to 13.

15. The host cells according to claim 14 which are animal cells.

16. The host cells according to claim 15, wherein the animal cells are mammalian cells.

17. The host cells according to claim 15, wherein the animal cells are insect cells.

18. A process for producing a target protein which comprises using the protein expression vector or the host cells according to any one of claims 1 to 18.

19. A target protein which is obtained by the process according to claim 18.

20. A process for producing a recombinant fusion protein comprising an amino acid sequence of a target protein which comprises using the protein expression vector or the host cells according to any one of claims 1 to 18.

21. A recombinant fusion protein comprising the amino acid sequence of the target protein obtained by the process according to claim 20.

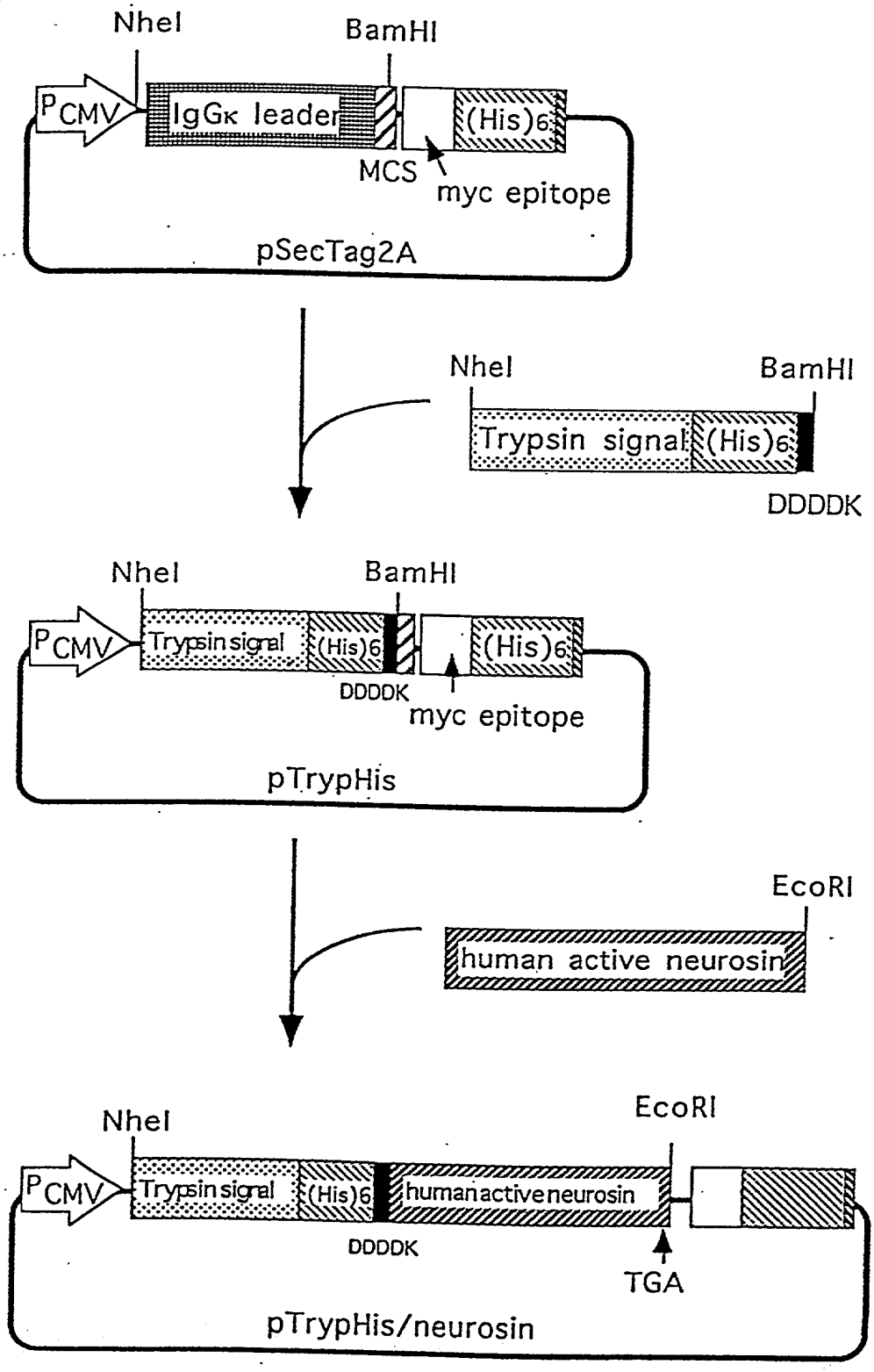
22. A process for producing a target protein which comprises retaining the recombinant fusion protein according to claim 21 with a substance capable of recognizing Tag and/or an epitope in said recombinant

fusion protein, liberating the recombinant fusion protein from the substance to purify it, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant fusion protein, followed by collecting the released target protein.

23. A process for producing a target protein, which comprises retaining the recombinant fusion protein according to claim 21 with a substance capable of recognizing Tag and/or an epitope in said recombinant fusion protein, and releasing the target protein by reacting said purified recombinant fusion protein with an enzyme capable of recognizing the cleavable site within said recombinant fusion protein, followed by collecting the released target protein.

24. A target protein is obtained by the process according to claim 22 or 23.

Fig. 1


NheI
BamHI
PCMV
IgGκ leader
(His)6
MCS
myc epitope
pSecTag2A
NheI
BamHI
Trypsin signal
(His)6
DDDDK
NheI
BamHI
PCMV
Trypsin signal
(His)6
(His)6
DDDDK
myc epitope
pTrypHis
EcoRI
human active neurosin
NheI
EcoRI
PCMV
Trypsin signal
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human active neurosin
DDDDK
TGA
pTrypHis/neurosin

Fig. 2

1; cell extract

2; culture supernatant

Fig. 3

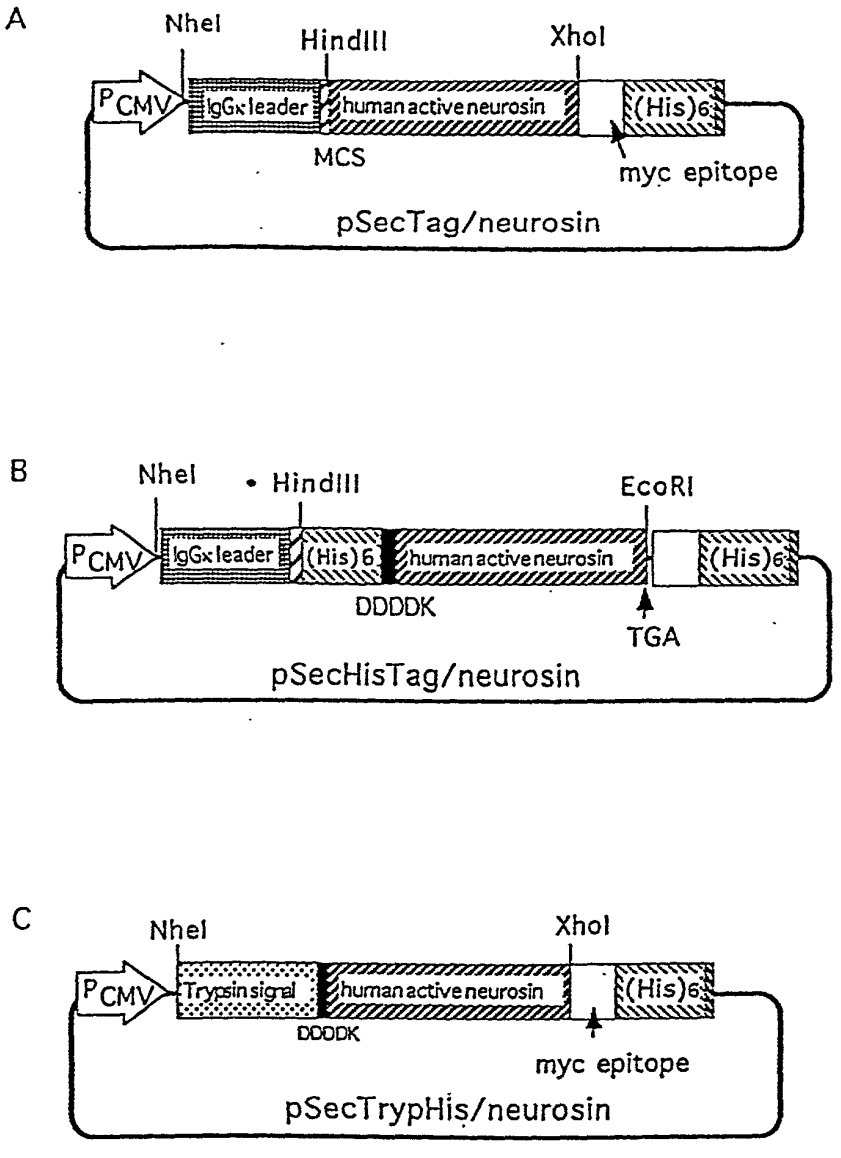

A
NheI
HindIII
XhoI
PCMV
IgGκ leader
human active neurosin
(His)6
MCS
myc epitope
pSecTag/neurosin
B
NheI
HindIII
EcoRI
PCMV
IgGκ leader
(His)6
human active neurosin
(His)6
DDDDK
TGA
pSecHisTag/neurosin
C
NheI
XhoI
PCMV
Trypsin signal
human active neurosin
(His)6
DDDDK
myc epitope
pSecTrypHis/neurosin

Fig. 4

1; pSecTag/neurosin
2; pSecHisTag/neurosin
3; pSecTrypHis/neurosin

Fig. 5

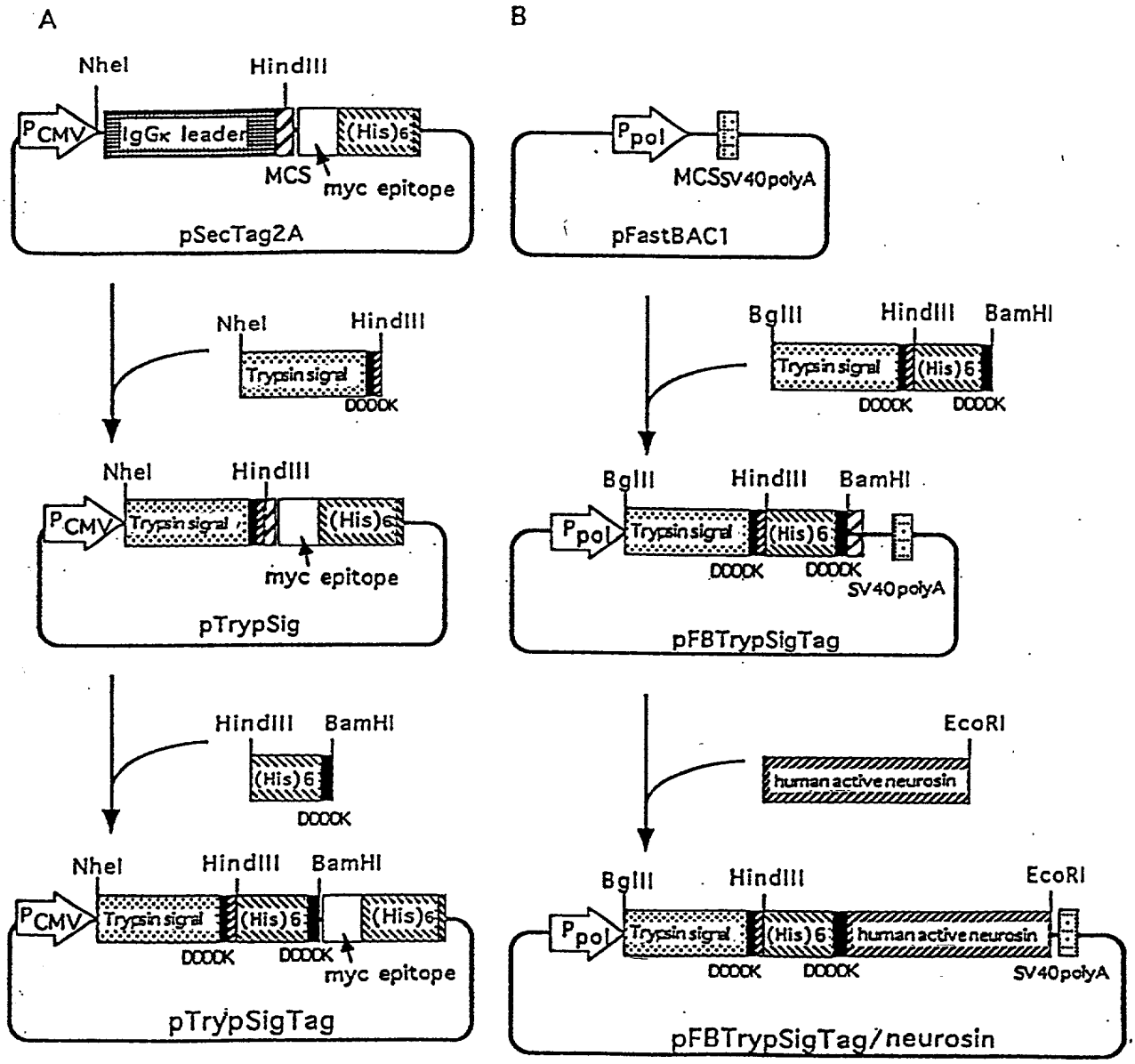
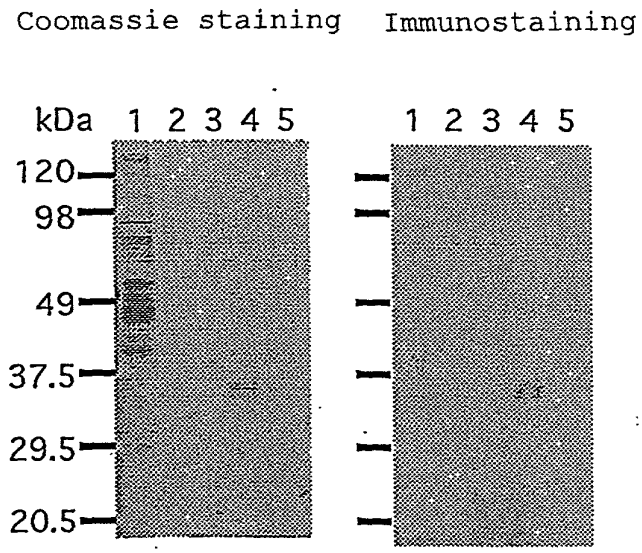

A
B
NheI
HindIII
PCMV
IgGκ leader
(His)6
MCS
myc epitope
pSecTag2A
Ppol
MCSSV40polyA
pFastBAC1
Trypsin signal
DDDDK
BglII
BamHI
pTrypSig
pFBTrypSigTag
SV40polyA
EcoRI
human active neurosin
pTrypSigTag
pFBTrypSigTag/neurosin

Fig. 6

1; culture supernatant

2; cell extract

Fig. 7



1; pass

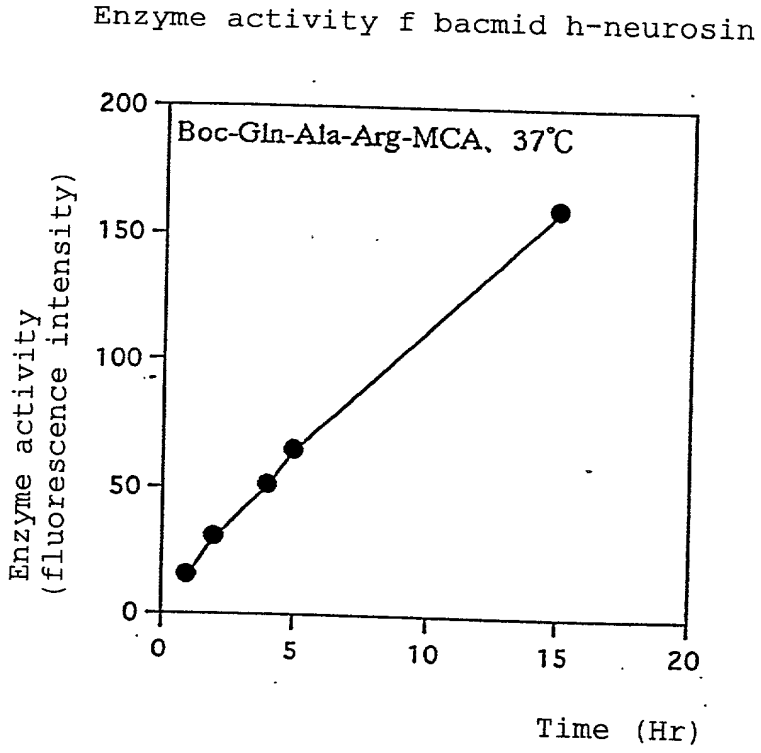
2; 5 mM imidazole

3; 10 mM imidazole

4; 100 mM imidazole

5; 500 mM imidazole

Fig. 8


Enzyme activity f bacmid h-neurosin
Boc-Gln-Ala-Arg-MCA、37℃
Enzyme activity
(fluorescence intensity)
200
150
100
50
0
0
5
10
15
20
Time (Hr)

Page 1 of 2 Pages [x] Original [ ] Supplemental Atty. Docket:

Combined Declaration for Patent Application and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROTEIN EXPRESSION VECTOR AND USE THEREOF

the specification of which (check one)

[ ] is attached hereto;

[ ] was filed in the United States under 35 U.S.C. §111 on _____, as U.S. Appln. No. _____*; or

[x] was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international (PCT) application, PCT/JP99/06474; filed 19/11/1999, entry requested on _____*; national stage application received U.S. Appln. No. _____*; §371/§102(e) date _____* (* if known)

and was amended on _____ (if applicable).

(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 and 365 of any prior foreign application(s) for patent or inventor's certificate, or prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked and have also identified below any such application having a filing date before that of the application on which priority is claimed:

(Number)	(Country)	(Day Month Year Filed)	[x] YES	[ ] NO
331515/1998	Japan	20/11/1998		
			[ ] YES	[ ] NO

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or prior PCT application(s) designating the U.S. listed below, or under §119(e) of any prior U.S. provisional applications listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information as defined in 37 C.F.R. §1.56(a) which occurred between the filing date of the prior application and the national filing date of this application:

(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the following registered practioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

All of the practioners associated with Customer Number 001444

Direct all correspondence to the address associated with **Customer Number 001444**; i.e.,

BROWDY AND NEIMARK, P.L.L.C.
624 Ninth Street, N.W.
Washington, D.C. 20001-5303
(202) 628-5197

The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from AOYAMA & PARTNERS as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

Page 2 of 2 Pages

Atty. Docket:

Title: PROTEIN EXPRESSION VECTOR AND USE THEREOF

U.S. Application filed _____, Serial No. _____

PCT Application filed Nov. 19, 1999, Serial No. PCT/JP99/06474

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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POST OFFICE ADDRESS: [illegible] 275-3, Tsutsui-cho, Yamatokoriyama-shi, Nara Japan		
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3) Katsuya KOMINAMI	Katsuya Kominami	Apr. 17, 2001
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4) Nozomi YAMAGUCHI	Nozomi Yamaguchi	Apr. 17, 2001
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POST OFFICE ADDRESS: 285-79, Shingoryoguchi-cho, Teramachinishi-iru, Kuramaguchi-dori, Kita-ku, Kyoto-shi, Kyoto Japan		
FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
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FULL NAME OF SIXTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENT		CITIZENSHIP
POST OFFICE ADDRESS		
FULL NAME OF SEVENTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENT		CITIZENSHIP
POST OFFICE ADDRESS		

ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING. ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO EXECUTION. NO ALTERATIONS CAN BE MADE AFTER THE DECLARATION IS SIGNED. ALL PAGES OF DECLARATION MUST BE SEEN BY ALL INVENTORS.

SEQUENCE LISTING

<110> Fuso Pharmaceutical Industries Ltd.

<120> Protein expression vector and use thereof

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<151> 1998-11-20

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Val Phe Leu Val Arg Ala Val Ile His Pro Asp Tyr Asp Ala Ala Ser
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65 70 75 80

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35 40 45

Val Phe Leu Val Arg Ala Val Ile His Pro Asp Tyr Asp Ala Ala Ser
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35 40